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STUDIES ON LENTIVIRUS INFECTION OF MACROPHAGES

WEI-CHENG LEE

This thesis is submitted as part of the course requirements for the degree of

Doctor of Philosophy at the University of Edinburgh

1994



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Declaration

The experiments and composition of this thesis are, unless otherwise state, my own work. No part of this work has been, or is being submitted for any other degree, diploma or other qualification.

Wei-Cheng Lee (March, 1994)

ABSTRACT OF THESIS (Regulation 3.5.10)

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Title of Thesis STUDIES ON LENTIVIRUS INFECTION OF MACROPHAGES

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Several aspects of the interaction of macrophages and maedi visna virus (MVV) were undertaken: viral replication, phenotype, phagocytosis and antigen presenting function of macrophages after MVV (EV1) infection.

MVV replication in monocyte-derived-macrophages (MDM) showed viral budding sites both at cytoplasmic and vesicular membranes. In contrast, viral budding sites predominantly occurred at the cytoplasmic membrane of skin fibroblasts, whilst virus accumulated in vesicular lumens of MVV-infected alveolar macrophages (AM). Many intracytoplasmic type A (ICA) particles accumulated in the cytoplasm of MDM and AM infected with EV1.

Expression of MHC class II, MHC class I, CD4, CD8, LFA-1 and VPM32 antigen on MDM infected *in vitro* was unaltered by 5 days after MVV infection ($P > 0.05$). *In vivo* MHC class I, class II (DQ & DR) and LFA-1 expression on AM from MVV infected sheep with lung lesions was greatly increased compared to uninfected sheep ($P < 0.05$). A significant decrease in the CD4 : CD8 ratio in bronchoalveolar lymphocytes was also found in the same group.

The phagocytic activity of macrophages after MVV infection was also studied both *in vivo* and *in vitro*. There was a decrease in the phagocytic activity for RBC ($P < 0.05$) and yeast by MVV-infected MDM after 5 days post infection, but the FcR expression of MDM assayed by erythrocyte rosetting (ER) did not show a significant difference between MVV and mock infected MDM. *In vivo*, there was no significant difference in ER, phagocytosis of RBC and *P. hemolytica* by monocytes between MVV-infected and control sheep. However surface binding and phagocytosis of opsonized *P. hemolytica* by AM from MVV infected sheep without lung lesions was significantly increased compared to uninfected sheep ($P < 0.05$), but this increase was not seen in ER and phagocytosis of RBC by AM in the same group. In contrast the ER, phagocytosis of RBC and *P. hemolytica* by AM from sheep with lung lesions was slightly lower, but not significantly different from uninfected sheep.

A defect in antigen presenting function of MDM *in vitro* was also found 3-5 days after MVV infection by using ovalbumin and PPD specific T cell lines as responding cells.

MVV-specific cytotoxic lymphocytes were activated by autologous MVV-infected skin fibroblasts, AM and MDM. MVV-infected MDM, AM and skin fibroblasts could be specifically lysed by MHC-specific and CD8⁺ cytotoxic lymphocytes (CTL). In addition, macrophages either infected or non-infected were non-specifically lysed by lymphokine-activated killer cells.

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ABBREVIATIONS

The following abbreviations have been used throughout this thesis.

| | |
|--------------|---|
| AC | accessory cell |
| ACC | antibody-dependent complement-mediated cytotoxicity |
| ADCC | antibody-dependent cell-mediated cytotoxicity |
| AIDS | acquired immunodeficiency syndrome |
| AM | alveolar macrophage |
| APC | antigen presenting cell |
| ARC | AIDS related complex |
| BAL | bronchoalveolar lavage |
| BSA | bovine serum albumin |
| CMI | cell mediated immunity |
| CAEV | caprine arthritis-encephalitis virus |
| CD | cluster of differentiation |
| Ci, μ Ci | Curie, microCurie |
| CPE | cytopathic effect |
| Con A | concanavalin A |
| CPM | count per minute |
| CSF | colony stimulating factor |
| CTL | cytotoxic T lymphocyte |
| DNA | deoxyribonucleic acid |
| DMEM | Dulbecco's Modified Eagles Medium |
| DMSO | dimethylsulphoxide |
| EDTA | ethylenediamine tetra-acetic acid |
| ER | erythrocyte rosette |
| FACS | fluorescence activated cell sorter |
| FcR | Fc receptor |
| FCS | foetal calf serum |

| | |
|-------------------|--|
| FITC | fluorescence isothiocyanate |
| g, mg, µg, ng | gram, milligram, microgram, nanogram |
| G-CSF | granulocyte-CSF |
| GM-CSF | granulocyte-macrophage-CSF |
| HBSS | Hanks' balanced salts solution |
| HEPES | N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid |
| HIV | human immunodeficiency virus |
| ³ HTdr | tritiated thymidine |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| KD | kiloDaltons |
| LAK | lymphokine activated killer |
| LPS | lipopolysaccharide |
| M, mM, µM | molar, millimolar, micromolar |
| Mab | monoclonal antibody |
| M-CSF | macrophage-CSF |
| MDM | monocyte-derived-macrophage |
| MF | mean fluorescence intensity |
| MHC | major histocompatibility complex |
| MVV | maedi visna virus |
| NK | natural killer |
| NMS | normal mouse serum |
| OVA | ovalbumin |
| PAGE | polyacrylamide gel electrophoresis |
| PBL | peripheral blood lymphocyte |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffer saline |

| | |
|--------------------|--|
| PBA | PBS with 0.1% bovine serum albumin |
| PBST | PBS with 0.05% tween 80 |
| PE | phycoerythrin |
| PHA | phytohaemagglutinin |
| PI | propidium iodide |
| PPD | purified protein derivative |
| RNA | ribonucleic acid |
| mRNA | messenger RNA |
| RPMI | Roswell Park Memorial Institute |
| SCP | sheep choroid plexus |
| SDS | sodium dodecyl sulfate |
| SEM | scanning electron microscopy |
| SIV | simian immunodeficiency virus |
| SK | skin cell |
| TCID ₅₀ | 50% tissue culture infectious dose |
| TEM | transmission electron microscopy |
| TNF | tumor necrosis factor |
| Tris | 2-amino-2-hydroxymethylpropane-1, 3-diol |

CHAPTER 1

INTRODUCTION

1.1 Introduction

Maedi visna virus (MVV), the prototype lentivirus, causes progressive interstitial pneumonia and encephalitis in sheep (Petursson et al., 1976). The disease was first described as a slow virus disease of sheep in Iceland during the period 1930-1950 (Sigurdsson, 1957; reviewed in Zink et al., 1987). The name given to MVV was derived from the syndromes it caused. Maedi, in Iceland, is a pneumonia of sheep, whilst, visna means wasting, which is due to an encephalitic lesion (Sigurdsson, 1952 & 1954 & 1957; Sigurdsson & Palsson, 1958). In North America the disease was described as ovine progressive pneumonia (OPP) and was first reported in 1915 (reviewed in Zink et al., 1987). Zwögzerziekte is the name used to describe the disease in Holland (Narayan & Clements, 1989).

MVV causes a persistent infection and replicates slowly in the host. Most infections are subclinical with the development of progressive inflammatory lesions in many organs eventually leading to symptoms. The lesions are characterized by infiltrations of mononuclear cells (Cutlip et al., 1979; Georgsson et al., 1990). MVV shares many biological similarities to human immunodeficiency virus (HIV) and other animal lentiviruses, such as gene structure, development of antigenic variation, persistent infection in macrophages, evasion of immune surveillance, and a chronic progressive course of disease (Narayan & Clements, 1989). The lentiviruses of humans, macaques and cats cause severe immunodeficiency in their natural hosts. The loss of CD4⁺ lymphocytes leading to opportunistic infection, neoplastic changes, and death of the host has been well documented in HIV infection, but this type of immunodeficiency is not seen with lentiviruses which infect ungulates (Narayan & Clements, 1989).

Animal models are important for research in AIDS. The simian and feline infection models most closely resemble HIV. As with other lentiviruses, including HIV, macrophages are predominant target cells in MVV infection. Lentivirus replication in macrophages may modify the non-specific cellular defence functions

of the host which could result in the failure to clear the virus and other infections. Therefore, studies on MVV/macrophage interaction may help towards a better understanding of current studies of HIV infection, particularly in viral persistence and the accessory cell function of virus infected macrophages (Narayan & Clements, 1989).

1.2 Epidemiology

Maedi visna is a common lentiviral disease of sheep in most parts of the world, except Australia and New Zealand where it has been kept out due to the restricted importation of sheep (Narayan & Clements, 1989). The disease occurred in an epidemic form in Iceland following the introduction of subclinically infected sheep from Europe during the 1930s (Sigurdsson, 1953). It led to the loss of least 150,000 sheep between 1939 and 1952 but was finally eliminated from Iceland by the slaughter of infected animals and the control of sheep importation (Sigurdsson, 1957). Explosive epizootic infections of the type seen in Iceland are rare. Usually few animals become overtly ill, and these are adults that were infected 2-3 years previously (Narayan & Clements, 1990).

The disease has shown two major symptoms: visna, a neurological disorder which mainly occurs in Icelandic sheep and maedi, a chronic progressive interstitial pneumonia. Certain breeds, such as Border Leicester sheep in USA, Texel in Holland and Icelandic sheep are more susceptible to the disease than Columbia sheep (Narayan & Clements, 1989; Cutlip et al., 1986). This suggests that genetic factors in the host regulate the extent of viral gene expression in specific cell types and organs.

The epidemic nature of the outbreak in Iceland is thought to be related to three main factors (Narayan & Clements, 1989):

(a) Host: The Icelandic sheep were of a different genotype than the imported carrier of infection.

(b) Management: MVV is principally transmitted via the horizontal route (Pearson et al., 1989). Evidence has shown that the prevalence of maedi-visna correlates with the length of exposure of lambs to MVV-infected ewes (Houwens et al., 1983). It has also been shown that transmission of MVV from ewe to lamb occurs through ingestion of contaminated colostrum or milk (reviewed in Cutlip et al., 1988). Vertical transmission can occur, but is rare (Cutlip et al., 1981). However MVV may also spread by exhalation of infected cells which then pass from one infected animal to another. In Iceland during the winter most sheep are kept inside in crowded conditions which increases the opportunity of nose-to-nose contact with easy transmission of virus via nasal exudates.

(c) Coinfection with a secondary agent: During the outbreak of maedi visna in Iceland, the sheep also developed sheep pulmonary adenomatosis (SPA) which produces profuse quantities of pulmonary exudate facilitating the transmission of disease (Narayan & Clements et al., 1989).

1.3 Classification and Morphology of Retroviruses

The family Retroviridae has been divided into 3 subfamilies, Lentivirinae, Oncovirinae, and Spumavirinae (Dahlberg et al., 1988). The classification is based on morphological distinctions, the host cell lines in which virus will grow, and immunological techniques. However, when viral genome and viral gene expression are used as criteria, the viruses form two categories, simple and complex retrovirus, which can be further divided into seven subgroups (Coffin, 1990; Cullen, 1992). The seven subgroups of retroviruses are: C-type retroviruses group A, C-type retroviruses group B, B-type retroviruses, D-type retroviruses, Lentiviruses, T-cell leukemia viruses, and Spumaviruses (Coffin, 1990; Cullen, 1992).

MVV is the prototype of the lentiviruses, even though HIV is now the most studied lentivirus. Besides MVV and HIV, the lentiviruses also include caprine arthritis encephalitis virus (CAEV), equine infectious anaemia virus (EIAV), feline

immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), and bovine immunodeficiency virus (BIV) (Dahlberg, 1988).

Bernhard (1960) first identified and classified retrovirus particles as A, B, C, and D particles in mouse tumor cells depending on the difference in morphology shown by electron microscopy. In his classification, type A particles, 60 - 90 nm in diameter, consisted of 2 forms, intracytoplasmic and intracisternal particles. Both forms are double-shelled with electron-lucent centers. Originally, it was not known that type A particles are the core of the type B retroviruses. Type A and type B particles are 2 different morphological forms associated with the genus *Oncovirus* B, generally termed type B viruses. Type B particles such as mouse mammary tumor virus (MMTV), which bud at the cell membrane with a complete nucleoid to form extracellular particles, have an eccentric nucleoid and prominent surface spikes on their outer envelope. Type C viral particles, such as murine leukemia virus (MuLV), bud at the cytoplasmic membrane with a crescent shape, but with no intermediate intracytoplasmic form as noted in type B viruses. The extracellular viruses contain a central nucleoid and lack surface projections and spikes. Type D viruses, such as Mason-Pfizer monkey virus (MPMV), have a similar morphology to type B viruses, but the mature virions contain short surface spikes (Fine et al., 1978; Gelderblom et al., 1991). HIV buds at the cytoplasmic membrane with a crescent shape and there is no intermediate form in the cytoplasm of virus-infected cells. The morphology of HIV during budding is similar to type C particles (Palmer et al., 1985; Munn et al., 1985; Orenstein et al., 1988).

Mature MVV, 70-100 nm in diameter, contains an envelope which is covered with short spikes and an eccentrically located, cone-shaped nucleoid, 30-40nm in diameter (Thormar 1961; Coward, et al., 1970), which is composed of viral *gag* protein. The spikes are formed by the envelope glycoprotein of MVV (Dubois-Dalcq et al., 1976). Virus maturation and release from the cytoplasmic membrane are very similar to type C viral particles. There is no intermediate form in the

cytoplasm, when MVV is grown in sheep choroid plexus cells (SCP) (Coward et al., 1970; Macintyre et al., 1973; Weiland et al., 1980). But Filippi et al (1982) report that infectious intracellular ribonucleoprotein complexes of MVV are found in the cytoplasm of SCP, which may indicate that intracellular infectious particles may exist, but do not aggregate in the cytoplasm and therefore are overlooked under TEM observation. In addition, ICA particles, probably an intermediate form, are also seen in the cytoplasm of MVV-infected lamb testicular cells (Takemoto et al., 1971). Furthermore, ICA particles are also reported in CAEV, which is the most closely related lentivirus to MVV, when it replicates in Himalayan tahr ovary cells (Dahlberg et al., 1981). Therefore, although MVV like HIV appears to be a type C retrovirus it may also produce intracytoplasmic intermediates like these seen in type B viruses.

1.4 Viral Genome and Viral Replication

1.4.1 Entry

Virus binding to the cellular receptor is the first step in infection. The binding of external gp120 of HIV virions to CD4 molecules on the T cell or macrophage membrane is initial phase of the infection for HIV (Earl et al., 1992). After binding, the viral envelope protein may be cleaved by a cell membrane associated protease or undergo a conformational change to expose gp41 which leads to virus membrane fusing with the cell membrane and nucleocapsid entry into the cytoplasm (reviewed in Levy, 1993). However, the processes between virus binding to the cell surface and entry into the cell are not clear. The process does not require high CD4 concentrations on the cell surface as monocytes or cultured macrophages have a low density of CD4 molecules on their cell membrane (Crowe et al., 1987; Kazazi et al., 1989; Collman et al., 1990), but are still infected by HIV, *in vivo* and *in vitro*. The virus can be blocked by anti-CD4 antibody (Collman et al., 1990). Moreover, some CD4⁻ cells derived from brain or gut can also be infected by HIV

(reviewed in Levy, 1993). Hence HIV entry into CD4⁺ cells may not be via the CD4 molecule. In reference to this it has been shown that HIV infection of macrophages is markedly enhanced by adding sera from certain HIV infected patients (Takeda et al., 1988). Antibody mediated enhancement of HIV infection of macrophages was not inhibited by monoclonal anti-CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) and was mediated by the FcγRIII, not FcγRI or FcγRII, expressed on macrophages (Takeda, et al., 1988; reviewed in Meltzer et al., 1990a). Complement receptors expressed on macrophages have also been implicated in binding HIV to the cell (Robinson et al., 1990). These phenomena suggest that immunological responses to HIV may facilitate the infection in the host.

To date the receptor on macrophages for MVV is not clear. MHC class II on macrophages may act as a component of a cellular receptor for MVV (Dalziel et al., 1991), although a 50K protein has also been implicated on fibroblast cells (Crane et al., 1991). MVV has also shown antibody mediated enhancement of infection of macrophages in a similar way to HIV (Jolly et al., 1989).

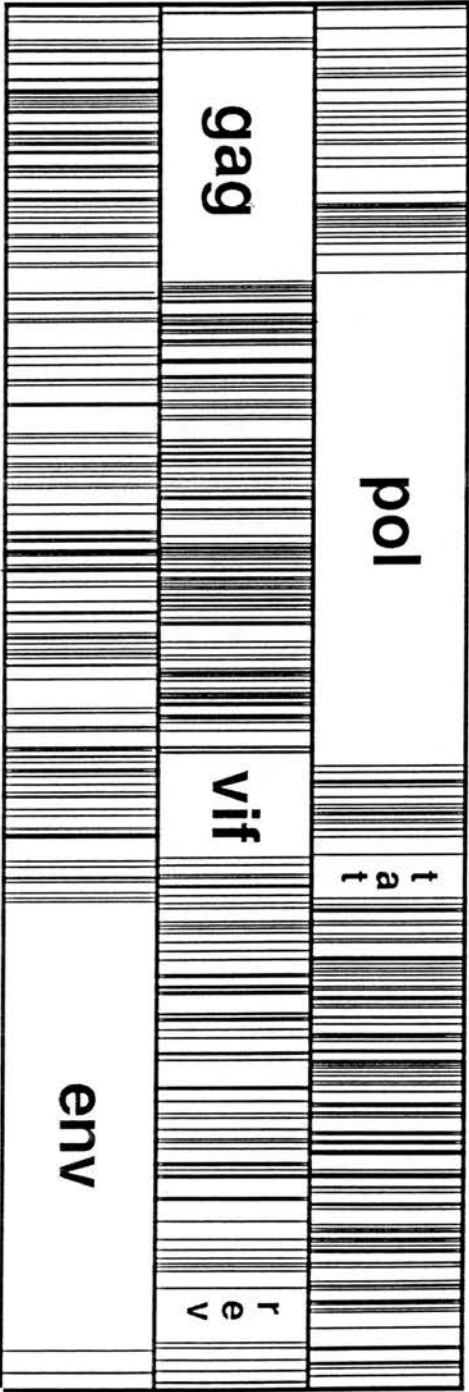
1.4.2 Virus genome and gene expression

MVV, like other retroviruses, has a diploid RNA genome about 9,200 bp (Sonigo et al., 1985; Pyper, et al. 1986; Sargan et al., 1991). It encodes three basic structural genes, *gag*, *pol*, and *env* (5' to 3') flanked by terminal repeats which contain promoter and enhancer elements. Thus the overall genomic organization of MVV RNA is RU5-*gag-pol-Q-env*-U3R (figure 1.1). In addition, the virus contains several small ORFs: *tat*, *rev* and *vif* (open reading frame Q) (Sonigo et al., 1985; Pyper et al., 1986; Sargan et al., 1991), which are thought to code for regulatory proteins. These small ORFs are located between the *pol* and *env* gene and between *env* and the 3' terminal (Sargan et al., 1991) (figure 1.1).

Figure 1.1

Genome organization of EV1 (Sargan et al., 1991). Stop codons in each reading frame of the EV1 sequence are indicated by vertical lines. Open reading frame Q is now thought to be the MVV equivalent of HIV *vif* (Sonigo et al., 1985; Cullen, 1992).

FIGURE 1.1



After the penetration of the virion core into the cytoplasm, viral replication is initiated. The start of the first strand synthesis occurs just downstream of the 5' terminal where a t-RNA molecule is bound to the primer binding site. The reverse transcriptase carried in the virion begins adding deoxynucleotides to the t-RNA primer. The direction of the nucleotide additions is from 3' to 5' (on genomic RNA) to form the first strand of DNA(-) using both copies of the genome in the virus. The original genomic RNA is digested by the RNase activity of RT allowing synthesis of the second strand of DNA(+) from the DNA(-) template. The use of the two genome copies allows duplication of the terminal region to give the long terminal repeat (LTR) found in the provirus. The genomic sequence of proviral DNA is U3-R-U5-*gag-pol-env*-U3-R-U5 where U3-R-U5 is a LTR (Clements et al., 1979).

Generally, retroviruses have a strong requirement for dividing cells for replication. These dividing cells presumably provide optimal conditions for the synthesis of viral DNA and RNA and integration of proviral DNA. In contrast, lentiviruses have the ability to replicate in non-dividing cells (Thormar, 1963). MVV DNA replication predominantly occurs in the nucleus of infected cells (Haase et al., 1982) rather than in the cytoplasm as occurs in oncogenic retrovirus infection (Weiss et al., 1982). During replication of oncogenic retroviruses in tissue culture, the circular form of provirus is predominant (Varmus, 1982). The majority of MVV DNA in infected tissue culture is as linear duplexes (Harris et al., 1984) with only a low level of circular forms (Clements & Narayan, 1981., Harris et al., 1984). Integrated viral DNA is the most efficient template for viral RNA transcription in the retroviral system (Varmus, 1982). But in MVV the linear non-integrated DNA may also contribute to the transcription of viral RNA (Somasundaran & Robinson, 1981).

For replication of proviral DNA into genomic RNA and mRNA the retroviruses use transcription signals present in the proviral LTR (reviewed in Clements et al., 1990). All messages are initiated at the beginning of the 5' R region

(the cap site) and transcription proceeds through the viral genome to the poly A signal in the R region of the 3' LTR (Narayan & Clements, 1989). These processes are carried out by cellular RNA polymerase (reviewed in Dahlberg, 1988). It is known that viral gene expression is highly restricted in monocytes where only a low percentage of cells are expressing viral RNA as detected by *in situ* hybridization. Again only a small percentage of these cells express detectable viral protein (Gendelman et al., 1985). However with monocyte differentiation and maturation much higher levels of viral RNA as well as viral proteins can be detected (Gendelman et al., 1985 & 1986), which may indicate that cellular transcription factors in activated macrophages are necessary for activation of viral transcription. This may be important in the shift of the viral life cycle from restrictive to productive infection.

1.4.3 Regulatory genes

Besides *gag*, *pol*, and *env* genes, lentiviruses also contain some small ORFs which regulate viral replication (Hess et al., 1985 & 1986; Steffy and Wong-Staal, 1991). In early transcription viral regulatory gene products including *tat* and *rev* proteins are expressed in HIV-infected cells (Cullen et al., 1989 & 1992). The *tat* protein in HIV-infected cells is essential for viral replication by activating viral RNA transcription via the viral LTR and also enhancing translation at a post-transcriptional level (Feinburg et al., 1986; Steffy and Wong-Staal, 1991; Cullen, 1992). This results in the rapid accumulation of viral RNA in the infected cells for productive replication. In contrast, MVV *tat* only modestly enhances MVV transcription and appears to do so by directly or indirectly interacting with AP-1 and AP-4 DNA target sites located in the U3 region of the viral LTR (Hess et al., 1989). The *rev* gene localized in the nucleoli of infected cells is required for the functional expression of the unspliced and singly spliced mRNA's encoding *gag*, *pol*, and *env* gene products and also mediates the establishment of an equilibrium between viral

structural and regulatory protein synthesis (Steffy and Wong-staal, 1991; Tilley and Cullen, 1992). The function of *rev* regulatory protein is similar between HIV-1 and MVV (Tilley and Cullen, 1992). MVV *Q* has some homology to the HIV-1 regulatory gene *vif* (Sargan et al., 1991; Audoly et al., 1992) which may play a role in the maturation of viral particles (Cullen, 1992).

The *nef* gene of HIV-1 acts as a negative regulatory gene (Guy et al., 1990; Cullen, 1992) and may play a role in restricted expression from the HIV-1 LTR and viral replication (Cullen, 1992). *Nef* appears important *in vivo* where a deletion in *nef* probably leads to decreased virulence (Guy et al., 1990). *Vpr*, *vpu*, *vpt*, and *tev* accessory genes have also been defined in HIV (Steffy and Wong-Staal, 1991; Cullen, 1992), but these genes are not defined in MVV.

1.4.4 Regulation of viral gene expression in infected cells

The balance between latent and productive viral infection is regulated by a complex interaction between exogenous signals, host transcriptional protein, and viral regulatory proteins. The lentivirus LTR contains many regulatory elements including promotor and enhancer sequences (Temin, 1981; Small et al., 1989; Hess et al., 1986). The induction of HIV LTR activity in T cells can be mediated by phorbol esters, UV irradiation, antigens, anti-CD3 antibody, mitogens, and cytokines such as TNF- α , IL-6 and GM-CSF (McCune, 1991). Many of these factors use signal transduction pathways where the final step is mediated by the transcriptional activator, NF- κ B, (Lenardo and Baltimore, 1989). *In vitro*, it has been shown that HIV-infected myeloid cell lines, monocytes or macrophages cultured with GM-CSF or M-CSF will amplify RT activity and increase the number of viral particles released into the culture fluid (Folk et al., 1987; Gendelman et al., 1988; Kalter et al., 1991). This suggests that GM-CSF or M-CSF promotes monocyte maturation while indirectly affecting virus replication in the cells. TNF- α

increases expression and replication of HIV in a latently infected T cell line (Clouse et al., 1989; Matsuyama et al., 1989).

In addition, a number of DNA viruses, such as cytomegalovirus and herpes simplex virus have been shown to transactivate the HIV LTR when the same cells are infected (reviewed in McCune, 1991). These viruses are also commonly found in patients with AIDS or at risk from AIDS. The increase in viral expression due to these factors may play a role in the onset and progression of clinical disease.

In contrast to HIV, addition of PHA or IL-2 to cultures of infected sheep PBMC reduces MVV replication (Gorrell et al., 1992). IFN- α has been shown to suppress HIV replication in human monocytes (Poli et al., 1989; Kornbluth et al., 1989 & 1990; Gendelman et al., 1990). In MVV-infected sheep, virus replication in macrophages is markedly reduced by IFN- γ released from activated T cells (Narayan et al., 1985; Kennedy et al., 1985). It has been suggested that its mechanism of action is to inhibit the development of monocytes into macrophages.

1.4.5 Translation and viral structural proteins

About 20 polypeptides are incorporated into MVV particles (Lin and Thorman, 1979). The major components of the virion are the envelope glycoprotein and the *gag* proteins (Haase et al., 1974; Vigne et al., 1982). In all retroviruses the *gag* or *gag-pol* polyproteins can be thought of as single translation unit (Dahlberg, 1988). The message size of *gag-pol* is not different from *gag* messenger RNA and it is thought that in lentiviruses the *gag/pol* precursor is made by a frame shift from *gag* into the *pol* gene (Dahlberg, 1988). The levels of *gag-pol* precursor are much lower than the precursor levels of *gag*, in a ratio of around 20 : 1, *gag* : *gag/pol* (reviewed in Coffin, 1990). Both precursors are further digested by proteases which are of viral origin (Dahlberg, 1986). Pulse-chase experiments have been carried out to establish how processing occurs (Vigne et al., 1982).

It has been elucidated that the precursor *gag* protein, Pr55^{*gag*} is further cleaved into p16, p25 (p30), and p14 (Vigne et al., 1982; Houwers et al., 1989). P16, matrix protein may direct protein to sites of viral budding (Rein et al., 1986). It is acylated and probably accumulates on the internal face of the cell membrane and in the virion, it may contact both the viral envelope and p25 core. P25 is the major core protein of the virus. This protein and the reverse transcriptase of *pol* are the most highly conserved of retroviral proteins. P14 is a small basic nucleoprotein that contains a repeated motif of cysteines which probably bind and protect the RNA genome (Filippi et al., 1982; Sonigo, 1985).

The *pol* gene is expressed as a *gag-pol* polyprotein precursor, Pr150^{*gag-pol*} (Vigne et al., 1982), which is thought to be further cleaved into reverse transcriptase (RT), integrase/endonuclease, and protease. RT converts the viral genetic information from the RNA genome to the DNA provirus. Endonuclease/integrase integrates proviral DNA into cellular DNA. Protease cleaves *gag* protein as the virion buds (Vigne et al., 1982; Sonigo et al., 1985).

In contrast, *env* protein is synthesized from a singly spliced subgenomic mRNA from which the *gag* and *pol* coding regions have been removed (Dahlberg, 1988). In lentiviruses the *env* gene encodes a single large polypeptide varying from 115K to 160K which is highly glycosylated during synthesis (Sonigo et al., 1985; Vigne et al., 1982). In MVV the precursor *env* protein has a molecular weight of 150,000, gPr150^{*env*} (Vigne, 1982). The envelope polypeptide contains the basic amino acid sequence Arg-X-Lys-Arg which is present in all retroviruses (Sonigo, et al., 1985). The sequence represents the cleavage site of the envelope glycoprotein into an outer membrane protein, gp135 (or gp110), and transmembrane protein, gp41 (Vigne et al., 1982). The envelope glycoprotein, which is less conserved among lentiviruses than *gag*, contains many biologically important determinants, including the determinants of virus-cell receptor interaction, virus induced cell fusion (Sargan et al., 1991; Lifson et al., 1986a & b), and the epitopes for virus

neutralizing antibody (Scott et al., 1979). Antigenic drift of MVV in persistent infection of sheep is also due to the mutations within the MVV envelope glycoprotein (Scott et al., 1979; Stanley et al., 1987).

1.4.6 Viral assembly

Transmission electron microscopy (TEM) studies have revealed that assembling maedi visna virions are first seen as an electron dense, crescent-shaped patch at the cytoplasmic membrane. Both ends of the crescent meet to form a complete envelope and core and the virions are released from the cells (Thorman, 1961; Dubois-Dalcq, et al., 1976). The protrusion of the cell membrane appears to be initiated by attachment of capsid protein to a region of the cell membrane which is modified by the formation of clusters of globular units (Dubois-Dalcq et al., 1976). These globular units are most probably local concentrations of envelope protein in the cellular membrane.

The assembly and budding pattern of HIV virions differs between infected macrophages and lymphocytes. HIV infected T cells show hundreds of viral particles associated with the cytoplasmic membrane that indicate HIV assembles and buds only from the plasma membrane of infected T cells and there is no intracellular accumulation of mature or immature virus reported (Gendelman et al., 1989). However, HIV replication in macrophages shows few or no virions at the cytoplasmic membrane. Virus is localized almost exclusively in intracellular vacuoles of macrophages (Gendelman et al., 1989; Orenstein et al., 1988; Munn et al., 1985). HIV not only accumulates within these intracellular vacuoles, but also assembles and buds from the vacuolar membrane (Orenstein et al., 1988). Evidence also suggests that these vacuoles are derived from the Golgi complex (Orenstein et al., 1988). The typical CPE of syncytial formation in HIV infection is mediated by envelope glycoprotein (Lifson et al., 1986). Finally, these virus-containing vacuoles are released into extracellular areas by exocytosis or cellular lysis. Ten fold less

virus is released from HIV-infected macrophages into culture fluid than is released by an equal number of infected T cells (Meltzer et al., 1990b). This suggests that the amount of virus released from macrophages is greatly limited, but HIV-infected macrophages are supposed to represent the major reservoir of persistent infection in human. Studies of immunoprecipitation patterns show that virions assembled from macrophages are relatively lacking in gp120, the outer membrane envelope glycoprotein, compared to virions released from T-lymphocytes. This may affect cellular tropism and ability to form syncytia.

MVV assembly in sheep choroid plexus or skin fibroblasts and macrophages is very similar to HIV with its difference in budding between T-lymphocytes and macrophages. There are extensive budding sites at cytoplasmic membranes of MVV-infected sheep choroid plexus cells (Thormar, 1961; Weiland and Bruns, 1980), whereas viral particles accumulate in intracytoplasmic vacuoles of MVV-infected macrophages (Narayan et al., 1982). Whether there is a relative lack of gp135 to *gag* protein in virions assembled from MVV-infected macrophages, as noticed in HIV infection in macrophages, is still unclear.

1.5 Immune Response

1.5.1 Humoral immunity

After infection with MVV, sheep develop a virus specific humoral and cell-mediated immune response (CMI). Precipitating and complement fixing antibodies can be detected as early as 1 month post infection (Sihvonen et al., 1981), but 2-3 months or longer are usually required to develop neutralizing antibodies (Shivonen et al., 1981). However, neutralizing antibodies can be detected as early as 2-4 weeks in some experimentally infected sheep (Griffin et al., 1978). These antibodies are not consistent to any one viral antigen which may be due to variation in different viral strains (Larsen et al., 1982a), but both precipitating and

complement fixing antibodies remain at high levels through the course of disease (Gudnadottir & Kristinsdottir, 1967).

Of the 20 MVV proteins noted by Lin & Thorman (1979), the *env* and *gag* gene products are the major antigenic proteins (Houwens & Scharke, 1987). Agar gel precipitation methods have also indicated that antibodies to gp135 are major components of the antibody response in MVV-infected sheep (Klein et al., 1985). However only IgM and IgG₁, never IgG₂ responses, have been detected in the sera of MVV infected sheep (Reyburn, 1992a). Antibodies to gp120 from HIV seropositive individuals have been shown to mediate anti-HIV antibody-dependent cell-mediated cytotoxicity (ADCC) (Lyeryl et al., 1987; Blumberg et al., 1987). However, no MVV-specific ADCC activity can be detected in antiserum from MVV infected sheep. This may be due to the fact that IgG₂ is the ADCC antibody in sheep and there is no detectable IgG₂ anti-MVV in sera (Reyburn, 1992a).

The *gag* proteins of MVV especially p25, which is highly conserved among virus isolates (Sargan et al., 1991), elicit strong antibody responses during infection which are often used as an index of infection (Houwens & Nanta, 1989). The role of this antibody is not clear. However viral core protein (p24) is expressed on the cell surface of HIV-1-infected cells (Laurent et al., 1989) which would allow antibodies against *gag* proteins to be involved in ADCC or antibody-dependent complement mediated cytotoxic (ACC) activity against infected cells (Rook et al., 1987).

The effectiveness of these antibodies in reducing lentivirus infection is not clear. It has been shown that non-neutralizing antibodies can aggregate viral particles on the cell surface (Dubois-Dalcq et al., 1979) which would inhibit their spread. But antibody and virus may form an immune complex which could enhance viral infectivity by allowing entry of the monocyte/macrophage via Fc receptors (Takeda et al., 1988). High concentrations of antibody-positive serum also inhibit viral replication in tissue culture (Takeda et al., 1988). However it has been shown that the affinity of neutralizing antibody for MVV is lower than that of virus for

cells (Kennedy-Stoskopf & Narayan, 1986) which suggests a possible mechanism of how virus escapes from neutralizing antibodies and spreads *in vivo*. Furthermore, MVV is able to escape neutralizing antibodies via another mechanism, antigenic drift of the *env* proteins. Here new variants cannot be recognized by antibodies induced to the original MVV isolate (Clements et al., 1980; Narayan et al., 1978).

Neutralizing antibodies and ADCC also play an important role in the control of HIV infection (Merigan et al., 1991). In HIV, low or absent serum neutralizing antibodies correlate with a poor prognosis of disease (Scheppeler et al., 1988). Moreover, evidence shows that sera from healthy HIV seropositive individuals has a significantly higher level of ADCC activity compared to sera from patients with AIDS (Rook et al., 1987). The HIV envelope is the major target for humoral antibody responses. Several conserved domains localized on the envelope gp120 and the external portion of gp41 are primarily involved in antibody neutralization of virus (Matthews et al., 1986; Ho et al., 1987). However, persistent infected macrophages have a relative lack of expression of the viral envelope glycoprotein, gp120, on their surface (Meltzer et al., 1990b) which may assist in evasion of ADCC by infected cells. Anti-viral antibody may contribute to depletion of CD4⁺ lymphocytes as gp120 bound to the surface of lymphocytes via CD4 can cause lysis of the lymphocytes via ADCC but not ACC (Lyerly et al., 1987).

1.5.2 Cell-mediated immunity

The CMI response to MVV appears earlier than the humoral immune response. Lymphoproliferative responses can be detected as early as 1 to 7 weeks after sheep have been experimentally inoculated with MVV, but these reactions are then lost (Griffin et al., 1978; Larsen et al., 1982a; Sihvonen, 1981). The decrease in CMI responses sometime after MVV infection is very similar to other viral infections and is generally associated with clearance of virus. After acute MVV infection, there are some cells (macrophages) which contain proviral DNA and viral

RNA, but few cells express viral antigen (Gendelman et al., 1985). Perhaps not enough antigen is expressed to maintain detectable levels of sensitized cells in the blood. However, when followed for three years after infection, sheep showed a transient and irregular CMI response in the blood (Larsen et al., 1982b). A more recent report indicates that CMI can be persistently detected in the blood of experimentally and naturally infected sheep using purified viral or *gag* antigen. The major proliferative response is mediated by CD4⁺ lymphocytes (Reyburn et al., 1992b).

CMI responses are well known in HIV infection. Specific HIV antigen induced lymphoproliferative responses can be detected in HIV patients without symptoms (Reddy et al., 1987), but lymphoproliferative responses to mitogens or specific recall antigens are greatly decreased in patients with generalized lymphadenopathy or AIDS (Pinching et al., 1991). The suppression of lymphoproliferative responses correlates to the depletion of CD4⁺ lymphocytes, decreases in CD4⁺ lymphocyte function, and progressive destruction of dendritic cells (DC) (Pinching et al., 1991). DC and Langerhan's cells have a major antigen presenting function, particularly in primary responses (Harding et al., 1988). Decreased MHC class II expression on these antigen presenting cells (APC) has been noted in HIV infected patients and is associated with a severe impairment of antigen presenting function (Eales et al., 1988; Macatonia et al., 1989 & 1990; Meyaard et al., 1993). In contrast, CD4⁺ lymphocytes are not target cells of MVV and the lymphoproliferative response to mitogens, BCG or OVA are not significantly suppressed in MVV-infected sheep *in vitro*, when compared to normal control animals (Larsen et al., 1982a; Dr. P. Bird, personal communication). There is also no significant change in the CD4/CD8 lymphocyte ratio in peripheral blood mononuclear cells (PBMC) of MVV-infected sheep (Dr. P. Bird; personal communication). Mild immune suppression of MVV-infected sheep has also been reported (Myer et al., 1988), but most studies have no evidence for gross

immunosuppression associated with MVV infection. *In vivo*, it has been shown that MVV-infected sheep have a decreased delayed-type hypersensitivity response to PPD and primary antibody response to OVA (Dr P. Bird, personal communication). These results suggest that, *in vivo*, an immune defect is at the macrophage level. However, concurrent infection with the virus causing sheep pulmonary adenomatosis or pasteurellosis is often reported in research and field cases (Cutlip et al., 1979; Markson et al., 1983). This suggests that immunosuppression may occur in some MVV-infected sheep, particularly in lungs with pulmonary lesions.

Development of the humoral immune response and CMI response, particularly in the generation of specific cytotoxic lymphocytes (CTL) is very important in the hosts recovery from various viral infections. Precursors of MVV-specific MHC class I restricted CD8⁺ CTL which can be activated by culturing with IL2 and virus, show specific cytotoxic activity on autologous MVV-infected skin fibroblasts (Blackaws et al., 1994). However, *in vivo*, macrophages are the major target cells of MVV. The MVV-specific, MHC class-I restricted recognition of autologous infected macrophages has only been reported once (Kennedy-Stopskopfs, 1989a).

In contrast, HIV-specific MHC class I restricted CTL are directed against various HIV-infected targets which express viral antigens including proteins coded by the *env*, *gag*, and *pol* genes (Langlade-Demeyen et al., 1988; Riviere et al., 1989; Nixon et al., 1988) and other regulatory genes (Walker et al., 1989; Riviere et al., 1989). Moreover, MHC class II restricted, *gag* or *env* specific CD4⁺ cytotoxic lymphocytes have been reported in HIV infected patients or healthy HIV seronegative volunteers who were primed with recombinant *env* protein (Littaua et al., 1992; Orentas et al., 1990., Curiel et al., 1993). There have also been reports of HIV envelope specific cytotoxicity, which is not restricted by MHC antigens and may not be mediated by T cells (Riviere et al., 1989).

1.5.3 Innate immunity

Besides humoral and cell-mediated immune responses, both of which play a major role in acquired host defense, the body also requires a variety of cells and factors to deal with immediate antigenic insult. Monocytes and natural killer cells are armed to perform these non-specific activities (Taylor and Cohen, 1992).

Both clinical and experimental data suggest that NK cells mediate defence against viral infection and malignancies, including HIV infection (Bandyopadhyay et al., 1990; Merigan et al., 1991). NK cells constitute a small population of lymphocytes in blood that display a large, granular morphology. These cells are CD3⁻, CD2⁺ and CD16⁺ (Taylor and Cohen, 1992). The number of circulating NK cells is not significantly decreased in HIV-infected patients, including patients with AIDS (Katzman and Lederman, 1986). However, a decreased NK activity has been documented in HIV infection, especial those patients with AIDS-related complex (ARC) or AIDS when compared to levels in normal individuals (Rook et al., 1985; Katzman and Lederman, 1986; Poli et al., 1985b; Ljunggren et al., 1989; Scott-Algara et al., 1992). But, culture *in vitro* of NK cells from HIV infected individuals with IL-2 can restore cytotoxic activity (Rook et al., 1985). Hence, the decrease in NK activity is thought to be related to the depletion of CD4 cells in HIV-infected patients with AIDS or ARC. NK cells show significant cytotoxic activity against HIV-infected lymphoid cells (Ruscetti et al., 1986). There is no data documenting the role of NK cells in MVV infection.

Activated macrophages have been found to play an important role in resistance to some viral infections (Sissons and Oldstone, 1980; Kohl, 1991). However, in lentivirus infections, monocytes play a dual role as they not only act as a reservoir of virus, but also as effector cells and antigen presenting cells for the host. Hence, there is the interesting situation of whether monocyte/macrophages can lyse lentivirus-infected monocyte/macrophages. However, there is no evidence

documented that lentivirus-infected monocyte/macrophages are lysed by other macrophages using their inherent cytotoxic function or by ADCC mechanisms.

1.5.4 Evasion of the immune surveillance

After viral infection, both the host's nonspecific (NK, macrophages, IFN) and specific immune response are induced to eliminate the invading virus and destroy virus infected cells to allow the host to recover from infection (Rouse and Babiuk, 1979). However, lentiviruses can evade immune surveillance and persist in host cells. A number of mechanisms allow this immune evasion: proviral DNA integration and latency, restricted replication states in different maturation stages of macrophages, low affinity of neutralizing antibodies, cell to cell spread of virus, and antigenic variation (Narayan and Clements, 1989).

Integration of a viral genome into the host cell genome is an effective way to establish persistence in the host and when the DNA is truly latent, avoid immune recognition. It is thought that circular DNA is the precursor for integration into host DNA, however most MVV proviral DNA exists as a linear duplexes, with only low levels of the circular form (Harris et al., 1984). Hence integration of MVV into the host cell genome appears to be limited. *In vivo*, proviral DNA has been detected in low levels in promonocytes of the bone marrow and monocytes in blood (Gendelman et al., 1985). A regulatory protein encoded by the *nef* gene of HIV, which can inhibit HIV-1 LTR-specific gene expression and viral replication (Cullen, 1992), is thought to be involved in the initiation of latency. There is no *nef* gene in MVV but there may be a *nef*-like domain in the *rev* gene (Sargan et al., 1991).

Antigenic variation of MVV in persistently infected sheep is another feature of lentivirus infection (Narayan et al., 1978). Over a long time virus undergoes progressive antigenic variation, mainly within the *env* gene. In one infected animal serologically distinct virus could be isolated late in the course of the disease which

was not efficiently neutralized by sera obtained shortly after infection (Narayan et al., 1978). The infected animal subsequently developed neutralizing antibodies against these variants. The mutations in these isolates were located within 2 kilobases of the 3' terminus of the genome, which is within the *env* gene giving rise to amino acid substitutions within the glycoprotein (Clements et al., 1980).

Other factors including viral infection of macrophages, low affinity of neutralizing antibody (section 1.7.1), and LV-IFN (section 1.7.3) which also limit viral replication. These factors directly or indirectly inhibit viral protein expression on the cell surface which allows evasion of the host immune response.

1.6 Pathology

The onset of MVV disease is insidious. After infection virus persists for several months or years in the host with increasing symptoms resulting in progressive deterioration in health and finally death (Cutlip et al., 1988). There are two major clinical signs and pathological lesions in MVV-infected sheep. The first is a chronic interstitial pneumonia where clinically the sheep show shortness of breath and later dyspnea, especially after exercise. Affected lungs become heavy, enlarged and firm with multiple grey areas (Cutlip et al., 1979; Oliver et al., 1981a & b; Lairmore et al., 1988b). Some of them may show a concurrent infection with other pulmonary diseases (Cutlip et al., 1979; Markson et al., 1983; Myer et al., 1988) or adenomatosis (Markson et al., 1983; Myer et al., 1988). Histopathologically, classical maedi is characterized by marked thickening of interalveolar septa with smooth muscle hyperplasia and accumulations of lymphoid cells into follicular-like structures, particularly in peribronchial areas (Cutlip et al., 1979; Oliver, et al., 1981a&b). The histopathology of sheep lungs infected with both bacteria and MVV consists of a combination of lymphoproliferative interstitial pneumonia and bronchopneumonia (Lairmore et al., 1988b). The complexity of lesions resembles that found in pediatric and adult AIDS with opportunistic

pulmonary infections which are a major cause of death in humans with HIV infection (Joshi et al., 1986; Marcheusk, et al., 1985).

MVV causes neurological symptoms which are characterized by posterior ataxia. The symptoms may persist and progress over several weeks to months leading to hind limb paralysis (Cutlip et al., 1988; Georgsson et al., 1990). Pathological lesions are distributed throughout the brain and spinal cord, particularly in white matter. The major early lesion of the spinal cord is inflammation around the central canal, whereas the most prominent changes in later stages are randomly scattered demyelinated lesions in the white matter (Petursson et al., 1976; Georgsson et al., 1989). The choroid plexus is frequently inflamed with the formation of lymphoid nodules (Petursson et al., 1976). Histopathological lesions include perivascular cuffs with mononuclear cells, predominantly lymphocytes and plasma cells (Oliver et al., 1981a). In severe cases, demyelination, microglial nodules and multinucleated giant cells are present, especially in white matter (Petursson et al., 1976). In contrast to the pulmonary lesions, only a small proportion of MVV infected sheep develop clinical neurological lesions (Oliver et al., 1981a).

Neurological abnormalities are very common in HIV patients with AIDS (Wigdahl et al., 1990). The predominant cell type in the brain infected with HIV is the monocyte/macrophage. It is thought that the virus enters the brain through infected monocytes which release monokines, enzymes and chemotactic factors, which may be toxic to neurons and leads to infiltration with inflammatory cells. Another mechanism may involve direct virus infection of nerve cells that results in either direct cell killing or alteration in cellular metabolic function (Wigdahl et al., 1990).

MVV causes inflammation in the mammary glands with diffuse infiltration of mononuclear cells in the interstitial tissue of the glandular parenchyma and nodules of lymphocytes around glandular ducts (Cutlip et al., 1985; Lujan et al.,

1991; Watt et al., 1992a). MVV also induces arthritis, predominantly in carpal or tarsal joints which are thickened by an accumulation of mononuclear cells and villous proliferation of the synovial membrane (Cutlip et al., 1985; Oliver et al., 1981a; Lairmore et al. 1988b; Watt et al., 1992a). Studies on the mediastinal lymph node of MVV infected sheep show marked B lymphocyte proliferation, with mild proliferation occurring in T lymphocyte areas (Ellis et al., 1985c).

1.7 Pathogenesis

1.7.1 Cell tropism

MVV is highly tropic for the monocyte/macrophage lineage, such as macrophages in lung, brain, lymph nodes, bone marrow and spleen, but not in liver or connective tissue (Kennedy et al., 1985; Gendelman et al., 1985). The sheep lentivirus is thought not to be lymphotropic (Gorrell et al., 1992), however there is one report of rare cells in the CNS which are positively stained for MVV *gag* antigen which have the morphology of lymphocytes (Georgsson et al., 1989). It can not be excluded that these cells were MVV specific B cells with antigen attached to their immunoglobulin or perhaps monocytes which had similar morphology to lymphocytes in these tissue sections. All other reports have failed to find MVV proteins or RNA in lymphocytes. There has been one recent report that MVV may also infect dendritic cells in the blood at a higher frequency than monocytes (Gorrell et al., 1992). However, in this study the dendritic cells are recognized as non-B, non-T, non-adherent, and MHC class II positive cells. The depletion of adherent cells from PBMC by incubation of these cells in plastic flasks is not enough to completely remove monocytes (author's data) which are also MHC class II positive. Furthermore, dendritic cells do not survive in culture as well as monocytes, therefore more definitive proof is needed before it can be said conclusively that blood DC are infected by MVV.

Both CD4⁺ lymphocytes and macrophages are targets for HIV. Langerhan's and dendritic cells and some CD4⁻ cells are also infected by HIV (reviewed in Levy 1993). HIV isolates have shown different tropisms for lymphocytes or monocytes (Evans et al., 1987). Lymphotropic HIV strains do not infect monocytes, whereas, monocyte-tropic strains infect both lymphocytes and monocyte/macrophages (Collman et al., 1989). For SIV both lymphocytes and monocyte/macrophages are infected (McEntee et al., 1991; Banapour et al., 1991).

1.7.2 Lentivirus replication in macrophages

In situ hybridization for MVV indicates that infected cells are predominantly macrophages in different affected tissues, including lung, spleen, lymph node, and brain (Kennedy et al., 1985). However, only low levels of viral DNA or RNA (under 200 copies per cell) can be detected in monocytes/macrophages in these tissues. Moreover, only a few of these cells with viral RNA (about 1%) produce virus (Haase et al., 1976; Gendelman et al., 1985). Hence, the lesions caused directly by viral infection may be very limited. So why do the lesions persistently grow? A constant recruitment of latently infected promonocytes and monocytes from bone marrow into tissues (the Trojan Horse mechanism) which then differentiate and activate in the tissues, may allow constant low levels of viral antigen expression and so chronic active inflammation to persist (Gendelman et al. 1985; Peluso et al., 1985).

In vivo viral antigen or RNA expression in monocytes is very low, however, they become readily detectable after cultivation of monocytes or by co-cultivation of PBMC with sheep choroid plexus cells (Gendelman et al., 1986). This suggests that the very low frequency of infected monocytes may be an underestimate of the actual, *in vivo*, viral infection. In contrast, mature macrophages in target organs of the infectious animals have much higher levels of viral RNA and many of these have viral proteins (Gendelman et al., 1986; Narayan et al., 1983).

HIV can be successfully recovered from monocytes (adherent cells from blood) of HIV infected patients by coculture on activated lymphocytes during all stages of the disease, but recovery of virus from nonadherent cells (lymphocytes) has not been consistently successful from subclinically infected patients (Ho et al. 1989). Detection of proviral DNA in PBMC by polymerase chain amplification of DNA was at a frequency of 0.0025% (Ho et al., 1989). However, this frequency increases about 100-1,000 fold in patients with symptomatic disease (Ho et al., 1989; Schuitemaker et al., 1992b). These results suggest that during subclinical infection virus is predominantly associated with the monocyte/macrophage lineage. But, in later stages of HIV infection this switch to a lymphocyte infection with about 1 in 400 CD4⁺ T cells harboring virus (Ho et al., 1989). However, productively infected cells in early or late stages of disease do not exceed 0.001% (Harper et al., 1986).

In vitro, HIV-associated cytopathic changes in a monocyte monolayer are characterized by cell fusion and lysis of 20-40% of the cell population after 2 weeks in culture (Gendelman et al., 1988 & 1990). Furthermore, cultured monocytes from HIV-infected patients show a markedly lower ability to survive after 10 days of culture, when compared to monocytes cultured from a normal control (Muller et al., 1990) which may be due to HIV replication.

During HIV infection, the cells producing virus in tissues are not CD4⁺ lymphocytes but macrophages (Meltzer et al., 1990a; Gendelman et al., 1989). Langerhan's cells (LC) and dendritic cells (DC) play important roles in initiation and regulation of the immune response. Langerhan's cells found in the mucosa of the rectum and vagina may act as early target cells for HIV and vehicles to disseminate virus to the regional lymph nodes (reviewed in Gendelman et al., 1989) which leads to progressive follicular destruction (Armstrong and Horne, 1984; Cameron et al., 1992). DC function is also impaired in HIV infection (Macatonia et

al., 1990 & 1992). The destruction and impairment of LC and DC also causes immunodeficiency.

In vitro, MVV causes lytic infection of macrophages and sheep choroid plexus cells (SCP). The cytopathic effect (CPE) of MVV in SCP or sheep synovial cells is characterized by the rapid formation of syncytial cells and cellular lysis (Lairmore et al., 1987). More time is needed to produce these cytopathic changes in virus-infected macrophages and multinucleated giant cells are not as evident in virus-infected macrophages as in virus-infected fibroblasts. It is also known that MVV protein, including gp135 and gp41 are involved in cell fusion (Crane et al., 1988; 1991). CPE in virus-infected monocytes or macrophages are dependent on the virus strain (Lairmore et al., 1987) as well as the stage of maturation of the monocytes (Gendelman et al., 1986; Narayan et al., 1983). Three different CPE have been defined by MVV on fibroblasts and macrophages (Lairmore et al., 1987). Type 1 isolates produce a high virus titer and lysis of synovial cells, but replicate slowly and cause no fusion of AM. Type 2 isolates produce a low virus titer and less syncytia or no cell lysis in both synovial cells and AM. Type 3 isolates produce a high virus titer, cellular fusion and lysis in both synovial cells and AM. Furthermore, the pathogenicity of these different strain isolates correlates to the *in vivo* virulence in lambs (Lairmore et al., 1987).

1.7.3 LV-IFN

In MVV infection macrophages not only act as target cells for virus, but also process viral antigens and present them with MHC-class II molecules on the cell surface to initiate immune responses. The interaction of macrophages, lymphocytes and virus in culture results in the production of lentivirus-interferon (LV-IFN) (Narayan et al., 1985; Zink and Narayan, 1989). LV-IFN production can be blocked by monoclonal antibody to MHC class II (Narayan et al., 1985).

However the interaction of fibroblasts, virus and lymphocytes does not result in the production of LV-IFN (Narayan et al., 1985).

LV-IFN is a nonglycosylated protein of molecular weight 54-64 KD and is heat and acid stable (Lairmore et al., 1988a; Narayan et al., 1985). It has similar biological activities to IFN- α and IFN- γ . It inhibits the maturation of monocytes to macrophages *in vitro*, and also stops virus replication in monocyte/macrophages at the transcriptional level (Zink & Narayan, 1989). However, viral replication is not restricted in SCP cells in the presence of LV-IFN (Lairmore et al., 1988a). Macrophages treated with LV-IFN secrete relatively high levels of prostaglandin E2 (Narayan et al., 1988), which is potentially important in suppressing the functions of macrophages.

In vivo the high levels of MHC class II antigen expression seen on macrophages of lymph nodes, spleen, AM and macrophages isolated from inflammatory synovitis in MVV-infected sheep (Kennedy et al., 1985; Kennedy-Stoskopf et al., 1989b; Cordier et al., 1990; Harkiss et al., 1991) may be induced by LV-IFN activity. Infection of macrophages with MVV does not result in increased MHC class II expression, thus the virus does not appear to have any direct effect on expression of MHC class II molecules (Narayan and Zink, 1988). The increase in MHC class II expression on tissue macrophages may potentially increase the antigen presenting capacity of these cells (Kennedy et al., 1985) and so lead to the chronic stimulation of the immune response giving the inflammatory lesion seen in MVV infection. However, research has documented the suppressive effect on lymphoproliferative responses of activated AM due to secretion of nitric oxide (Stuehr and Marletta, 1987; Holt et al., 1985). Hence, AM, at least in mice, are recognized as suppressor cells, rather than cells which stimulate immune responses. Whether this is also the case with LV-IFN activated AM is unknown. Epidemiological evidence often shows concurrent infection of the lung with other

bacterial pathogens (Markson et al., 1983), which suggests that pulmonary immune function is suppressed in sheep with maedi.

Persistent LV-IFN activity may be the reason why AM in MVV infected sheep appear to be activated to spontaneously release fibronectin and increase their neutrophil chemotactic factor secretion (Cordier et al., 1990). This could accelerate the entrance of leukocytes into local tissues and enhance cell-mediated tissue damage. Other functions of AM may also be enhanced by IFN- γ activity: for example phagocytosis and their intracellular killing ability (Murray et al., 1987b). Local IFN production in infected target organs may be responsible for inhibition of viral replication and the chronic nature of the disease.

1.7.4 Immune mediated pathology

After MVV infection, sheep develop chronic active inflammation in many organs over a long latent period (Gendelman et al., 1985). With disease progression these inflammatory infiltrates destroy normal tissue architecture leading to impairment of normal function. However, the cause of lesions in MVV infection is very complex and may involve an immunopathological mechanism (either viral specific or autoimmune) and/or abnormal cytokine release from infected cells.

A major component of the pathology is immune mediated as it has been shown that treatment of sheep with cyclophosphamide or antiserum developed in the horse against sheep thymocytes suppresses the cell mediated immune response and reduces the development of the CNS lesions compared to untreated controls after MVV infection (Nathanson et al., 1976). Autoimmunity mediating lesion formation in MVV infection has not been reported, but the arthritis seen in MVV infection has similar lesions to rheumatoid arthritis and may be induced by heat shock proteins (Dr. G. Harkiss, personal communication).

Evidence drawn from studies on mediators released from AM indicates AM from MVV-infected sheep spontaneously release neutrophil chemotactic factor and

fibronectin which may induce pulmonary lesions including cellular infiltration and proliferation of fibrous tissue (Cordier et al., 1990). The AM from MVV infected lungs are therefore activated and so may release high levels of cytokines, eg IL-1 and TNF- α . There is also a massive increase in the number of AM in affected lungs which may amplify the lesion. *In vitro*, It has been suggested that ovine lentiviruses do not induce TNF- α secretions in AM culture (Ellis et al., 1991), although this work was poorly controlled. The local production of TNF- α stimulated by bacteria during dual pulmonary infection may be central to the pathogenesis of chronic ovine lung disease and lead to the cachectic state. Lentivirus-infected sheep with pulmonary lesions have significantly elevated levels of LV-IFN (see section 1.7.3) which activates AM and may also serve to amplify the local inflammatory response to promote tissue damage. In HIV infection it has been shown that inappropriate secretion of monokines (IL-1, IL-6 or TNF- α) by HIV-infected macrophages in the brain and lung (reviewed in Narayan & Zink, 1988; Fauci, 1988; Steffen et al., 1993; Breen et al., 1990) may induce both neurologic syndromes and tissue injury.

1.8 Functional Capacity of the Lentivirus-Infected Monocytes

Monocyte/macrophages secrete some 100 products categorized as enzymes, complement components, enzyme inhibitors, reactive oxygen intermediates, cytokines, hormones and coagulative factors (Nathan, 1986). These secreted products are involved in a wide range of biological functions including phagocytosis, intracellular killing and antigen presentation; all very important in host defense mechanisms.

Macrophages are now recognized as important cells in HIV infection. Data including membrane phenotype, chemotaxis, antimicrobial activity, phagocytosis and antigen-presenting functions have been reported in monocyte/macrophages from HIV-infected patients. However there is limited documentation of these areas in

MVV-infected sheep, even though the monocyte/macrophage lineage is recognized as the predominant target cells for MVV infection.

Most research shows that monocytes do not significantly change in number in HIV-infected patients (Smith et al., 1984), but there may be a decrease in monocyte numbers in the blood in late stages eg. AIDS. There are no reports of decreasing monocyte numbers in MVV-infected sheep. Surface molecules, including CD4, CD8 and FcR expressed on monocytes in HIV seropositive patients are not different from seronegative controls (Davidson et al., 1988). However MHC class II expression on monocytes from HIV-infected patients with AIDS is decreased when compared to seronegative controls, but a significant difference is not found in HIV patients without AIDS (Davidson et al., 1988; Heagy et al., 1984). A similar phenomenon is also found in AM from HIV patients with AIDS (Roy et al., 1987). The decreased MHC class II expression on monocytes or macrophages may be related to the severe depletion of CD4 lymphocytes in patients with AIDS. Expression of MHC class II molecules on antigen presenting cells is related to their function. Some groups have reported that the antigen presenting function of monocytes infected with HIV, *in vitro*, is decreased (Petit et al., 1988; Macatonia et al., 1989; Ennen et al., 1990), whereas, others showed no effect (Mann et al., 1990b). Defects in accessory cell function of PBMC were not observed in HIV infected people (Clerici et al., 1990). This is probably a reflection of the very low frequency of circulating monocytes which are infected. However, an increase in MHC class II expression has been found in AM from sheep with maedi (Cordier et al., 1990; Lujan et al., 1993) and macrophages isolated from carpal joints of MVV-infected sheep with inflammatory synovitis (Harkiss et al., 1991). The up-regulation of MHC class II expression on these macrophages may be mediated by LV-IFN activity (Narayan et al., 1985).

Monocyte chemotaxis in response to stimulation by N-formylmethionyl-leucyl-phenylalanine (f-met-leu-phe), C5a, endotoxin, *Giardia lamblia* and casein is

depressed below normal levels in HIV-infected patients with AIDS, particular in those patients with Kaposi's sarcoma or opportunistic infections, when compared to healthy control patients (Poli et al., 1985a; Smith et al., 1984). Evidence also indicates that serum taken from asymptomatic seropositive individuals may reduce chemotaxis by monocytes from normal individuals. These results suggest that the reduction in chemotactic activity seen in HIV infected patients was due to an inhibitory factor present in the serum. In contrast, there is an increase of neutrophil chemotactic attractant and fibronectin from AM in MVV-infected sheep (Cordier et al., 1990). These factors are closely related to the pathogenesis of pulmonary lesions. There are no significant differences in the levels of IL-2 and prostaglandin E2 (PGE2) production from pulmonary lymph node leukocytes from MVV-infected sheep or uninfected controls (Lairmore et al., 1988a).

Functional analysis of macrophages has been approached both *in vivo* and *in vitro* in HIV infection. Phagocytosis in monocyte/macrophages is of major importance and functions to remove and destroy invading pathogens. Fc and C3b receptors on the monocyte surface mediate these activities (Fanger et al., 1989). Non-specific phagocytosis of latex beads or infectious agents such as *Candida* or *Toxoplasma gondii* by monocytes from HIV-infected patients with AIDS is normal (Bender et al., 1988). However, receptor-mediated (Fc and C3 receptors) phagocytosis is impaired in HIV patients with AIDS, but not in those patients without the AIDS syndrome (Bender et al., 1988). Intracellular killing assays determine the ability of monocytes and macrophages to kill intracellular pathogens. This function of macrophages can be enhanced by treating cells with IFN- γ or TNF (Murray et al., 1987b; Bermudez and Young, 1988). Studies of the intracellular killing activity of monocytes both in steady state or after further *in vitro* exposure to IFN- γ from HIV patients have shown normal results (Murray et al., 1987b).

Some secretory products, which are related to the effector function of monocyte/macrophages, including H₂O₂ and TNF, show normal secretion levels or

appropriately increased levels after *in vitro* treatment of the cells with IFN- γ or lipopolysaccharide (LPS) (Murray et al., 1987a; Capsoni et al., 1992). TNF- α released in the supernatant of cultured peripheral blood monocytes from symptomatic HIV-1 infected patients is significantly higher than normal controls. IL-1 also shows a similar elevation in the symptomatic HIV-1 infected group, but does not reach statistical significance (Roux-Lombard et al., 1989). Both cytokines act as effector molecules to stimulate collagenase and collagen synthesis, and trigger IL-2 and IL-6 secretion (Dinarelli, 1991). These factors may be involved in the pathogenesis of HIV infection.

Analysis of tissue macrophages in HIV-infected patients shows a very different picture to that of the relatively normal blood monocyte population (Smith et al., 1984; Poli et al., 1985a). It has been shown that skin Langerhan's cells and dendritic cells are severely affected in HIV infection (Armstrong et al., 1984). Changes are characterized by condensation of nuclear chromatin and vacuole formation in the cytoplasm. HLA-DR and CD1 expression on these cells is markedly decreased by at least 50% (Belsito et al., 1984). These cellular changes increase in parallel with disease progression (Armstrong et al., 1984). Indeed, in late-stage disease there can be a complete loss of dendritic cells which are important for antigen presentation in lymph nodes (Cameron et al., 1987).

The results of phenotype and effector function of macrophages are often controversial and not well defined during HIV infection (reviewed in Gendelman et al., 1989). The variation in results may indicate that low numbers of circulating monocytes are infected and so there is no great alteration in monocyte function. In addition, monocytes which will eventually migrate into other tissues have a relatively short circulating half-life in blood (Beelen et al., 1989). Most importantly, perhaps, the long incubation times seen with HIV infection may introduce many confounding factors including drug therapy, secondary infections,

and of greatest effect, the depletion of CD4 lymphocytes, which will affect the results obtained in functional assays of monocyte/macrophages

AM play an important role in response to pulmonary disease. Immunosuppression is not a recognized feature of MVV infection. However, most research into the problem of immunosuppression is limited to the field of lymphoproliferative responses to mitogens or antigens which may not be the correct area for study. Perhaps what should be studied in MVV infection is the accessory cell functions of macrophages. Also the tissue from which the macrophages is derived (whether affected by MVV infection or not) could have an strong effect on the types of changes seen.

1.9 Aims of Project

Macrophages are predominant targets and reservoirs of lentiviruses, particularly for MVV and CAEV. Macrophages have a broad range of biological activities which are important in host defense mechanisms, particularly in phagocytic and antigen presenting functions. Study of the interaction of macrophages and virus (MVV) can be used not only for the pathogenesis of MVV infection, but also as a model of HIV/macrophage interaction.

The project presented here has looked at several aspects of the interaction of macrophages and MVV (EV1, British strain). These studies include firstly, the characteristics of MVV replication in macrophages (where viral assembly occurs in cell, at what stage in *in vitro* differentiation of macrophages is virus produced); secondly, whether MVV infection alters macrophage phenotype and effector functions (using erythrocyte rosetting and phagocytosis); and thirdly whether the antigen presenting function of macrophages for soluble antigen is changed after MVV infection. Finally, the ability of MVV infected macrophages to act as targets for cell mediated cytotoxicity was assessed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All chemicals were supplied by SIGMA Chemical Co., Poole, Dorset, England or BDH Chemical Ltd., Poole, England unless otherwise stated. Radiolabelled compounds were supplied by Amersham International plc., Amersham, Bucks, England. Tissue culture plasticware was supplied by GIBCO-BRL, Paisley, Scotland. Universals and centrifuge tubes were supplied by Bibby Sterlin Ltd.

2.2 Cell Lines and Cell Culture

2.2.1 Culture of skin fibroblasts

Sheep skin fibroblasts derived from biopsies as detailed in Bird et al., (1993) were cultured in Dulbecco's modified Eagle's medium (Gibco, BRL Paisley Scotland) containing 2mM L-glutamine, 100U penicillin per ml, 100 µg streptomycin per ml and 10% FCS (DME / 10%FCS) at 37°C, 5% CO₂.

Cell lines were detached using 0.05% (w/v) trypsin in 0.02% (w/v) EDTA in PBS and passed to other tissue culture flasks at 1:3.

2.2.2 Freezing and thawing of cells

1-2 x 10⁶ cells per ml, in an ice-cold mixture of 90% FCS and 10% DMSO, were frozen slowly by placing the cells in a polystyrene box filled with cotton wool at -70°C overnight and then the vials were transferred to liquid nitrogen. To resuscitate frozen cell lines, an aliquot was warmed quickly at 37°C, gradually diluted with warm DME / 10%FCS, and then centrifuge at 350 xg for 5 minutes. The cell pellet was suspended in DME / 10%FCS, incubated for 5 minutes at 37°C, and then pelleted again. Finally, the cells were resuspended in DME / 10%FCS and transferred into 25 cm² tissue culture flasks.

2.2.3 Monocyte/Macrophage Culture

2.2.3.1 Gelatin-plasma-coated flasks or plates

Gelatin-coated flasks or plates were prepared according to the method described by Jones et al., (1989). 2.5 ml or 0.5 ml of 2% sterile gelatin was added to each 25 cm² tissue culture flask or well of 24-well plates. After 1 hour incubation at 37°C, the gelatin was removed and the plates dried in a 37°C incubator. The dried flasks and plates could be stored at room temperature until use. Immediately before use, they were incubated with autologous plasma for 30 minutes at 37°C. Plasma was removed and the flasks or plates washed twice with PBS. These pretreated flasks or plates were then ready for the culture of monocytes.

2.2.3.2 Monocyte isolation and culture

Blood was collected in heparin-containing tubes (10 units for 1 ml blood). Buffy coat cells were obtained by centrifugation of the heparinized blood at 1,250 xg for 15-20 minutes at room temperature. Autologous plasma was collected and used for the gelatin-coated flasks or plates. The buffy coat was diluted in sterile phosphate buffered saline (PBS) (0.85% NaCl, 0.02% KCl, 27 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2), overlaid on Lymphoprep (Nyegaard, Oslo, Norway) and then centrifuged at 1,250 xg for 20 minutes at room temperature. Peripheral blood mononuclear cells (PBMC) were harvested at the interface and then washed three times (500 xg, 5 minutes) with PBS. The PBMC were finally suspended in RPMI-1640 medium containing 2mM L-glutamine, 20mM Hepes, 100 U penicillin per ml, 100 µg streptomycin per ml, and 5 x 10⁻⁵M 2-mercaptoethanol (2-ME) (RPMI) with 10% fetal calf serum (FCS) and 10% lamb serum (LS) (Gibco) (RPMI / 10%FCS/ 10%LS). The PBMC were counted and the concentration adjusted to 5 x 10⁶ cells per ml. Normally, 0.5 ml, 5 ml or 15 ml of this cell suspension was plated into each well of a 24-well plate, 25 cm² or 75 cm² flasks,

respectively. The cells were left for 1 hour or overnight at 37°C, 5% CO₂, depending on the experiment and the non-adherent cells were then washed off with warm RPMI / 2%FCS. Fresh RPMI/ 10%FCS/ 10%LS was added for continuous culture. The cultural medium was replaced every 5 days. After 5-7 days culture, most non-adherent lymphocytes could be removed as above and the cultured adherent macrophages (monocyte-derived-macrophages; MDM) were usually nearly confluent. Over 95% of these adherent cells were non-specific esterase (NSE) positive.

After 7 days in culture, normal cultured MDM showed proliferation and some detached to become suspended in the supernatant. The suspended cells could be passaged to other flasks where they became adherent and could be used in further experiments.

2.2.4 Alveolar macrophage (AM) isolation and culture

AM were collected by bronchoalveolar lavage (BAL) with cold Hank's Balanced Salt Solution (HBSS). The cells were pelleted by centrifugation at 700 xg at 4°C for 10-20 minutes, depending on the volume of BAL fluid. Cell pellets were washed with RPMI / 2%FCS three times. The cell number was counted and adjusted to 5×10^5 cells per ml in RPMI / 20%FCS. Specimens contaminated with blood or parasites were not used. Five ml of cell suspension were seeded into 25cm² flasks and then incubated at 37°C, 5% CO₂. Non-adherent cells were removed 2 hours later and the flasks re-fed with fresh medium. The purity of AM was over 95% as determined by NSE staining. AM were cultured in a similar manner to monocytes but with RPMI / 20%FCS

To harvest adherent MDM or AM, cultures were washed with PBS once and 2.5 ml of 5 mM EDTA in PBS was added to 25 cm² flasks. After 2-5 minutes incubation, the flasks were knocked to dislodge the cells and a rubber policeman

used to scrape off any adherent cells remaining. The cells were washed once with warm RPMI / 2%FCS and then used.

2.2.5 Non-specific esterase (NSE) staining (Hudson and Hay, 1989)

Cytospins or cells cultured on glass coverslips were fixed in cold 45% (v/v) formaldehyde, 25% (v/v) acetone in 0.1M phosphate buffer (pH 6.6) for 40 seconds. The fixed cells were washed with water and incubated with the substrate, 0.025% α -naphthyl butyrate developed in 0.067 M phosphate buffer, pH 5.8, which contained 0.12% (w/v) sodium nitrate and 0.12% (w/v) pararosaniline, for 40 minutes at 37°C. The cells were washed in running water and then counterstained with 0.4% methylene green for 5 minutes. Cells were classified as positive by the appearance of a brown color.

2.2.6 Giemsa Staining

Fibroblasts were fixed in cold methanol/acetone mixture (1:1) for 5 minutes and then treated with 1% potassium dichromate for another 30 minutes. The treated cells were further fixed by cold methanol/acetone mixture for 5 minutes. The cells were stained with Giemsa's solution (2 fold dilution made fresh) for 5 minutes. The stained cells were washed in running water, dried in air, and then mounted in DPX. Macrophages were stained as above but after washing in water they were incubated in Giemsa' buffer (0.1 M citric acid, 0.2 M disodium hydrogen phosphate pH 5.75) for 5 minutes, rewashed and mounted as above.

2.3 Virus

2.3.1 Virus isolates

Maedi visna virus (MVV), strain EV1, was isolated on skin fibroblasts from PBMC of a sheep with an acute maedi-like disease and has a limited passage history *in vitro* (Sargan et al., 1991). The Icelandic strain of MVV, 1514, was obtained

from J.E. Clements, The John Hopkins University School of Medicine, Baltimore, Maryland and has a long passage history in tissue culture. Skin fibroblasts were grown to 90% confluence in 175 cm² or 225 cm² flasks and then infected with EV1 at a low multiplicity of infection (0.1 TCID₅₀ per cell) in DME / 2%FCS. When cultures showed extensive cytopathic effects (5-7 days), the virus-containing supernatant was harvested, cellular debris removed by centrifugation at 1,500 xg at 4°C and stored, in aliquots, at -70°C. MVV was titrated on skin fibroblasts as described below.

2.3.2 Experimental infection and titration of MVV

For experimental infections, skin fibroblasts were infected with 0.5 TCID₅₀ per cell in DME / 2%FCS for 2 hours at 37°C and virus inoculum removed and replaced with DME / 2%FCS. Macrophages were infected with 1-2 TCID₅₀ MVV per cell in RPMI / 2%FCS for 2 hours, 37°C and the virus inoculum replaced with RPMI / 10%FCS.

Virus titer was measured by limiting dilution and detection of cytopathic effects on skin cells. Virus-containing supernatants were serially diluted in DME / 2%FCS and 50µl added to 2 x 10⁴ skin fibroblasts also in 50 µl DME / 2%FCS per well of 96-well flat bottom microtiter plates. Six replicates of each diluted supernatant were set up and plates incubated for 3-4 days at 37°C, 5% CO₂ then 100 µl of DME / 2%FCS was added to each well with a further culture for 3-4 days. On day 7 the cells were fixed in cold acetone : methanol (1:1) and then stained with Giemsa's solution (see above). CPE were determined by syncytial cell formation. The virus titers were expressed as the 50% end point of the tissue culture infective dose (TCID₅₀) as calculated by the Reed & Meunch method (1938).

2.3.3 Viral antigen preparation (for lymphoproliferation assay and Western blotting)

Skin fibroblasts were infected with MVV at 0.1 TCID₅₀/cell or AM at 1 TCID₅₀/cell and maintained in DME / 2%FCS or RPMI / 10%FCS until extensive cytopathic effects were observed. The culture supernatant and infected cells (cells removed by a rubber policeman) were collected and cells pelleted by centrifugation (10,000 xg) for 20 minutes at 4°C. The cell pellet was suspended in a minimum amount of PBS (equivalent to approximately 4 x 10⁷ cell per ml) and stored, in aliquots, at -70°C to be used in immunoblotting experiments. Viral particles were pelleted from the clarified supernatant by further centrifugation 100,000 xg, 2 hours or 10,000 xg for 16 - 18 hours at 4°C. The virus pellet was suspended in a small volume of PBS and stored, in aliquots, at -70°C to be used for immunoblotting experiments and lymphoproliferative assays.

2.4 Detection of Viral Antigens

2.4.1 Immunofluorescent antibody staining (biotin-streptavidin method)

Cultured MDM, AM or skin fibroblasts infected with MVV, either grown on glass-coverslips or cytopins prepared from cell suspensions, were fixed in 80% methanol in saline. Fixed cells were washed in PBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide (PBA) and then blocked with PBA containing 2% normal rabbit serum and 2% normal sheep serum (PBA/2%NRS/2%NSS) for 30 minutes. The blocked cells were stained with the monoclonal antibody (Mab), anti-gag p15 (1:5,000 of ascites), 415 (gift from Dr. D. Houwers), or anti-p25, 1D-10 (VPM70) saturated supernatant (Reyburn et al., 1992c) for 1 hour at room temperature. The cells were washed 5 times in PBS with 0.01% Tween-80 (PBST) and then stained with sheep anti-mouse Ig-biotin conjugate (1:400) in PBA for 1 hour. The cells were washed 5 times with PBST and then stained with streptavidin-FITC conjugate (1:2500) in PBA (Serotec) for

another 1 hour. After 5 washes with PBST, the stained cells were mounted with citifluor (Citifluor Ltd., London, UK) and examined under a UV microscope (Leitz Ortholux II). MVV-infected cells stained with a nonspecific mouse IgG_{2a} or uninfected cells stained with specific monoclonal antibody were included in the experiments as the negative controls.

2.4.2 Detection of viral antigen expressed on the cell surface and in the cytoplasm by flow cytometry (McSharry et al., 1990)

The presence of MVV antigen on the surface of infected cells was detected by indirect immunofluorescence antibody (IFA) staining carried out in Rhesus tubes. Infected and uninfected cells were cultured and harvested as described before then blocked with PBA/2% NRS/2% NRS for 30 minutes at 0°C. The cells were further incubated with a monoclonal antibody (anti-p15 or anti-p25) at 0°C for forty minutes, washed three times with PBA and then incubated with FITC-conjugated, anti-mouse IgG F(ab')₂ (1:50 in PBA, Dakopatts Ltd) at 0°C for another twenty minutes. After three washes with PBA, cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry.

To detect internal viral antigen, the infected and uninfected cells were fixed in 80% methanol in PBS followed by three washes with PBA before blocking and incubation with monoclonal antibody as previously described.

2.4.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein

Resolving gels were prepared as 7.5%, 10% or 5%-20% linear gradient acrylamide gels (Acrylamide : N,N'-methylenebisacrylamide, 37:1) in 375 mM Tris HCl pH 8.7 and 0.1% SDS. Stacking gels were 3% acrylamide, 0.15% N,N'-methylenebisacrylamide with 125mM Tris HCl pH 6.8 and 0.1% SDS. The samples were mixed with sample buffer (0.1% bromophenol blue in 125 mM Tris HCl

pH 6.8 containing 20% (v/v) glycerol, 10% 2-ME (v/v), and 10% SDS (w/v) and then boiled for five minutes before electrophoresis. Five μg of protein as determined by Bio-Rad microtitre assay or 2×10^5 AM in 5 μl sample buffer were loaded in each track of a vertical slab gel (Bio-Rad Mini-protein II Slab cells) and then electrophoresed with running buffer (0.025 M Tris, 0.19 M glycine, 2 mM EDTA(Na_2), 0.1% SDS) at 180V for forty to sixty minutes.

After electrophoresis, the separated polypeptides in the PAGE gel were transferred to nitrocellulose membrane (Hybond C, Amersham) using a semi-dry electroblotter (Ancos, Denmark) in blotting buffer (0.192 M glycine, 25 mM Tris in 20% methanol) for two hours at 120 mA according to Kyhse-Andersen (1984). After blotting, the nitrocellulose membranes were blocked with 5% low-fat dried milk in PBS for thirty minutes, replaced with primary antiserum which was also diluted in 5% milk/PBS, and then left overnight at 4°C with shaking. The blots were washed with 6 changes of borate buffered saline (BBS) (0.1 M boric acid, 0.034 M sodium tetraborate, 0.075 M sodium chloride) / 0.05% Tween-80 and then incubated for 1 hour in an alkaline phosphatase conjugated secondary antibody (1:1,000) in 5% milk/PBS. The blots were washed with shaking in BBS/0.05% Tween-80 again and then developed with 0.02% nitro-blue tetrazolium and 0.01% 5-bromo-4-chloro-3-indolyl phosphate, 0.02 M MgCl_2 in 0.1 M Tris HCl pH 9.5 (Pluzek & Ramlau, 1988) for 15 to 30 minutes. The reaction was stopped by washing in water. Marker was separated before blocking and stained for 5 minutes in 0.5% Amido black and destained in 50% methanol, 5% acetic acid.

Gels were stained with Coomassie Blue (0.025% Coomassie Brilliant Blue G-250 dissolved in 20% methanol, 5% acetic acid and then filtered through Whatman No. 1 filter paper) for 15-20 minutes at room temperature and then destained with several changes of 20% methanol, 5% acetic acid in water.

2.5 Transmission Electron Microscopy (TEM)

Cultured MDM, AM, and skin fibroblast cells in 75 cm² flasks were infected with MVV at 1 TCID₅₀ (macrophages) or 0.5 TCID₅₀ (skin fibroblasts) per cell, respectively. On day 4, 6-7, or 9, depending on the morphological changes and CPE in the culture, infected and mock infected cells were gently scraped off the flask surface with a rubber policeman. The cells were washed once with PBS and then immediately fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), at 4°C for two hours. The fixed cells were washed with cacodylate buffer and then post-fixed in 1% osmium tetroxide for one hour at 4°C. The fixed cells were further dehydrated through a gradient of acetone, and then embedded in Araldite. Ultrathin sections (60 nm) were cut and stained with uranyl acetate and lead citrate. These stained sections were examined under a transmission electron microscope (Philips 400 Electron Microscope).

2.6 Scanning Electron Microscopy (SEM) Observation

For SEM examination, cells were cultured on glass coverslips in 24-well plates. Cells were infected with MVV (1 TCID₅₀ per macrophage or 0.5 TCID₅₀ per skin fibroblast) as described previously. Depending on the designated time and CPE formation in the cultures, coverslips were washed with PBS 5 times to remove non-adherent cells and cellular debris and then fixed in 3% glutaraldehyde in 0.1M cacodylate buffer at 4°C overnight. After three washes with cacodylate buffer, the cells were post-fixed in 1% osmium tetroxide for one hour at 4°C. The cells were rinsed in the same buffer, dehydrated in a gradient of acetone, and subsequently dried in a critical point dryer. The dried cells on the coverslips were mounted onto SEM stubs and then coated with evaporated gold palladium. The specimens were examined under a scanning electron microscope (Philips SEM 505).

2.7 Phenotypic Analysis of Cultured MDM, AM and PBMC

2.7.1 Cells and viral infection

MDM were cultured in 25 cm² flasks and infected with virus, 1 TCID₅₀ per cell on day 7, 9, 10 and 11 as previously described. AM were cultured for 1-3 days and then infected with MVV, 1 TCID₅₀ per cell. The cells were harvested by treatment with 5 mM EDTA in PBS as described in section 2.2.3 and MDM phenotype was analyzed on the same day.

2.7.2 Indirect immunofluorescent staining for flow cytometry

Cultured macrophages, AM or freshly prepared PBMC (section 2.2.3.2) were washed twice with PBA and then aliquoted into Rhesus tubes, 1 x 10⁵ macrophages or 1 x 10⁶ PBMC per tube. Cells were pelleted by centrifugation at 250 xg for 3 minutes, 0°C and then resuspended in monoclonal antibody for 40 minutes, 0°C. The panel of monoclonal antibodies used is shown in table 2.1.

Indirect immunofluorescent antibody staining used in flow cytometry was performed as follows: cells were pelleted and incubated in 25 µl of monoclonal antibody (saturated supernatant or ascites diluted in PBA) for 40 minutes at 0°C. The cells were washed three times with cold PBA and then stained with 25 µl anti-mouse IgG, F(ab')₂-FITC conjugate (1:50) (Dakopatts Ltd), for 20 minutes at 0°C. After three washes in cold PBA, 5,000 or 10,000 cells gated using FSC and SSC on macrophage or lymphocyte areas respectively were analyzed by flow cytometry using a Becton-Dickinson FACScan with Consort 30 version F software. Normal mouse serum (NMS, 1:500) stained cells were set up as background staining controls. The percentage of positive cells and mean fluorescence intensity (MF) were determined by using Lysys Version 1.62 and Consort 30 Version F.

TABLE 2.1 Monoclonal Antibodies to Sheep Leukocyte Antigens

| Monoclonal antibody | Isotype | Antigen | Specificity |
|---------------------|-------------------|------------------------------------|--|
| SBU-T4(1) | IgG _{2a} | CD4 | MHC class II restricted T cells |
| SBU-T8(1) | IgG _{2a} | CD8 | MHC class I restricted T cells |
| VPM8 (2) | IgG ₁ | Ig light chain | B cells |
| VPM19(3) | IgG ₁ | MHC class I | nucleated cells |
| VPM32(4) | IgG _{2a} | not defined | macrophages |
| VPM33(4) | IgM | not defined | B & T cells, monocyte/macrophages |
| VPM36(5) | IgG ₁ | MHC class II DQ- α chain | B & activated T cells, DC, monocyte/macrophages |
| VPM38(5) | IgG ₁ | MHC class II DR- α chain | B & activated T cells, DC, monocyte/macrophages |
| VPM65(6) | IgG ₁ | CD14 | monocyte/macrophages, DC, neutrophils |
| ST197(7) | IgG _{2b} | T19 | $\gamma\delta$ T cells |
| F10-150-39 (8) | IgG ₁ | LFA-1 | B & T cells, monocyte/macrophages |
| L180-1 (9) | IgG ₁ | LFA-3 | B & T cells, monocyte/macrophages |

1. Maddox et al., 1985
2. Bird et al., in preparation
3. Hopkins & Dutia, 1990
4. Gonzalez, 1988
5. Dutia et al., 1990
6. Gupta et al., in preparation
7. Mackay et al., 1989
8. Mackay et al., 1990
9. Hunig et al., 1985

2.7.3 Generation of concanavalin A (Con A) supernatant

Single cell suspensions were prepared from the mesenteric lymph node of sheep. The lymph node was teased apart with sterile forceps and then put through a stainless steel mesh. Single cell suspensions were collected and washed twice in RPMI / 2%FCS. Concanavalin (Con) A-supernatant was prepared by culturing of mesenteric lymphocytes, 1×10^7 /ml, with 5 µg Con A per ml in RPMI / 10%FCS for 2 days at 37°C, 5% CO₂. After centrifugation to remove cells, the supernatant was collected and stored, in aliquots, at -20°C.

In this study 50% Con A-supernatant in RPMI / 10%FCS was normally used to activate cultured MDM for 2-3 days.

2.8 Phagocytosis

2.8.1 Cells and virus infection

MDM were cultured in 24-well plates as described in section 2.2. The cultured MDM were infected with MVV, 1 TCID₅₀ per cell on day of 5, 7, 9 or 11 of culture. The phagocytic assay was performed on day 12 or 13 of culture.

2.8.2 FITC-labelled particles

The method described by Tuijnman et al (1990) was used to FITC conjugate particles. To label sheep RBC, a 5% suspension of sheep RBC in microtube was washed three times with PBS using a microfuge (Sanyo) at 3,000 rpm, 2 minutes to pellet the RBC and incubated with FITC at a final concentration of 100 µg/ml in PBS for 30 minutes at 37°C. The FITC labelled SRBC were washed twice in PBS and then opsonized with a pooled antiserum.

To label yeast (*Candida utilis*) (Buschmann et al., 1989), yeast were cultured in nutrient broth supplemented with 2% glucose in an incubated shaker at 30°C overnight. The cell suspension was washed three times with PBS by centrifugation (500 xg, 10 minutes at 4°C) and the pellet resuspended in PBS at a concentration of

4×10^8 yeast/ml and FITC stock solution (1 mg/ml) was added to a final concentration of 100 μ g/ml. The suspension was stirred at ambient temperature for 30 minutes and then washed in PBS three times. The pellet was finally suspended in PBS at 1×10^8 yeast/ml and then used in the phagocytic assay.

To label *Pasteurella hemolytica* (serotype A6) (kindly given by Dr J. E. Phillips), *P. hemolytica* was cultured on blood agar plates at 37°C overnight. Bacterial colonies were harvested by washing the plate with PBS and scraping off the bacteria with a rubber policeman. The bacterial suspension was washed once with PBS using a bench centrifuge (Beckman, Microfuge E) at 12,000 rpm for 30 seconds. The bacterial concentration, as determined by spectrophotometry (LKB, ULTROSPEC 4050), was adjusted to $OD_{550}=0.5$ which was the equivalent of $3-4 \times 10^8$ CFU per ml when checked by colony formation. Bacteria were labelled with FITC at a final concentration of 100 μ g/ml at 0°C for 30 minutes with mild mixing. The bacterial suspension was washed with PBS twice and resuspended to the original concentration for opsonization.

2.8.3 Opsonization

FITC-labelled sheep RBC were opsonized by a subhemagglutinating dilution (1:40 in PBS) of anti-SRBC antiserum developed in rabbits (kindly given by Dr. J. Hopkins) for 30 minutes at 37°C. The opsonized RBC were washed twice with PBS and then resuspended to 1% in RPMI / 2% FCS.

FITC-labelled *P. hemolytica* was opsonized with a rabbit antiserum to *P. hemolytica* (A6) (kindly given by Dr. W. Donachie; Moredun Research Institute, Edinburgh) The serum was titrated for optimum phagocytosis by macrophages and a 1:10 dilution of serum was used for further experiments. The FITC-labelled *P. hemolytica* was incubated with antiserum at 37°C for 30 minutes. The bacterial suspension was then washed twice with PBS and resuspended in RPMI / 2% FCS without antibiotics to the original concentration. A stock of FITC-labelled,

opsonized bacteria in 15% glycerol, RPMI with no antibiotics ($OD_{550}=0.5$), was stored at -70°C and used in phagocytic assays of fresh monocytes and AM from normal control and MVV-infected sheep.

2.8.4 Phagocytosis by cultured MDM

MDM were cultured and infected with MVV in 24-well plates as described before. The cultured MDM were washed with RPMI / 2%FCS to remove non-adherent cells. Assays were then undertaken on adherent cells. The cells were fed with 250 μl RPMI / 2% FCS containing 50 μl of FITC-particles and incubated for 1 hour at 37°C .

Non-ingested RBC were removed with PBS and any remaining on the outside of MDM hypotonically lysed with distilled water for 30 seconds, and immediately neutralized with 2x PBS. The cells were treated with 5 mM EDTA in PBS for five minutes and then gently scraped off with a rubber policeman. The cells were transferred into Rhesus tubes, washed with PBA and immediately analyzed by flow cytometry. MDM which had not been fed with FITC-targets were used as background controls.

In assays using bacteria and yeast, cells were washed with PBS 3 times and then harvested as described above. After being transferred into Rhesus tubes, cells were pelleted, suspended in 1% cold paraformaldehyde in PBS for 1 hour and analyzed by flow cytometry.

2.8.5 Erythrocyte rosette assay

Fresh AM or MDM (harvested with 5mM EDTA in PBS) were washed in cold PBA in Rhesus tubes as described before. The cell pellets were suspended in 50 μl of 1% FITC-labelled opsonized SRBC and then incubated at 0°C for 30 minutes. The samples were resuspended in PBA and immediately analyzed by flow cytometry.

2.8.6 Phagocytosis by fresh AM from MVV-infected and control sheep

AM were collected as previously described, but these cells were washed in RPMI / 2%FCS. One $\times 10^5$ AM were pelleted in Rhesus tubes and then suspended in 50 μ l of FITC-labelled particles. After 1 hour incubation at 37°C, non-ingested RBC were hypotonically lysed with distilled water for 30 seconds and neutralized with 2x PBS. After another 3 washes with PBA, the cells were analyzed by flow cytometry. The phagocytosis of *P. hemolytica* by AM was performed as previously described, but with frozen stocks of FITC-labelled, opsonized *P. hemolytica*. After 3 washes with PBS, the cells were fixed in 1% paraformaldehyde in PBS for an hour and then analyzed by flow cytometry.

2.8.7 Phagocytic and erythrocyte rosetting activity of monocytes

FITC-labelled, opsonized RBC and *P. hemolytica* (frozen stocks used) have been described earlier (section 2.8.2). A modified double-staining flow cytometry method was used to study phagocytosis by monocytes. Briefly, 1×10^6 PBMC in RPMI / 2%FCS pelleted in Rhesus tubes were fed with 50 μ l FITC labelled particles and then incubated for 1 hour at 37°C. The external RBC were hypotonically lysed by distilled water for 30 seconds and neutralized with 2x PBS. The cells were washed with cold PBA three times and then stained with the monoclonal antibody VPM65 (anti-CD14) followed by F(ab')₂ rabbit anti-mouse IgG R-phycoerythrin conjugate (1:50 Serotec) as described before (section 2.7.2).

For the erythrocyte rosetting assay PBMC were washed in PBA and then stained with the monoclonal antibody, VPM65, followed by the phycoerythrin conjugate. After 3 washes with cold PBA, the cells were incubated with opsonized RBC at 0°C for 30 minutes, resuspended in cold PBA, and then analyzed by flow cytometry. PBMC alone, PBMC fed with FITC-labelled particles, and PBMC stained with monoclonal antibody without FITC-labelled particles were used as background controls. Ten thousand cells were gated from the monocyte-enriched

area as determined by the FSC and SSC profile. Each treatment was performed in duplicate in an experiment.

2.9 Generation of Antigen-Specific T cell lines

2.9.1 Primed sheep

All sheep were adult Finnish Landrace crosses obtained from the Moredun Research Institute, Edinburgh, and were used to generate ovalbumin (OVA) and PPD-specific T cell lines. Sheep (1203T and 1275T) were primed with OVA, 1 mg in complete Freund's adjuvant (CFA), and boosted 3 weeks later with OVA, 1 mg in incomplete Freund's adjuvant (IFA). One week to 10 days after being boosted with antigen, the sheep were bled to obtain PBMC for generating OVA-specific T cell lines. These sheep were boosted with antigen once more in order to repeat this experiment.

Sheep 1275T after being primed with OVA in CFA also responded to purified protein derivative (PPD) (MAFF, Weybridge), probably due to the heat killed, dried *Mycobacterium tuberculosis* in CFA. It and sheep 1216V which had been primed with *Bacille Calmette-Guerin* (BCG), 5 human doses, 6 months previously, were boosted with 50 µg PPD in 1ml PBS, intradermally at 4 separate sites. The lymphoproliferative response to PPD was tested 7 days after boosting and PBMC in both sheep were then used to generate PPD-specific T cell lines.

2.9.2 Generation of antigen-specific T cell lines

PBMC were purified as previously described. Three x 10⁶ PBMC per well in RPMI / 10%FCS were stimulated with either 100 µg of OVA or 12.5 µg of PPD per ml in 24-well plates for 6-7 days at 37°C, 5% CO₂. This was the first antigen stimulation cycle. The cells were harvested, overlaid on Lymphoprep and centrifuged (750 xg, 10 minutes) to remove dead cells. Interface cells were washed two times in RPMI / 2%FCS before going on to IL-2 expansion cycle. For this



2×10^5 /ml viable lymphocytes in RPMI / 10%FCS medium supplemented with 10 U recombinant human IL-2 (from recombinant *E. coli*, N.I.B.S.C., Potter Bar) per ml were cultured for another 6-7 days. This cycle was sometimes prolonged to 9-14 days, depending on the lymphocytes appearance. Fresh rhIL-2 was added every 3-4 days. Viable cells were harvested over Lymphoprep as above. These two cycles were repeated 1-4 times more to obtain antigen specific T cell lines. For subsequent antigen stimulation cycles, 2×10^6 irradiated, autologous PBMC (3,000 Rad ^{137}Cs) per well were used as antigen presenting cells. Lines were harvested over Lymphoprep and 5×10^6 viable cells per aliquot were stored under liquid nitrogen as described in section 2.3.2. Some cell lines were cultured for 3-4 cycles before use.

Autologous MDM were often used as antigen presenting cells to stimulate the T cell lines instead of irradiated PBMC. Adherent cells in 24-well plates from which lymphocytes had been removed at the end of the first antigen stimulating cycle were used as a source of macrophages or MDM. The MDM were cultured in RPMI / 10%FCS/10% LS containing 50% Con A-supernatant (V/V) for 2-3 days at 37°C, 5% CO_2 and then used as antigen presenting cells in secondary antigen stimulating cycles with antigen and lymphocytes.

2.9.3 Lymphoproliferative assays

Freshly isolated PBMC (section 2.2.3.2) were cultured in RPMI / 10%FCS. One $\times 10^5$ PBMC in 100 μl RPMI / 10%FCS with 100 μl antigen in RPMI / 10%FCS were cultured in 96-well flat-bottomed plates at 37°C, 5% CO_2 . After 5 days, the cultures were pulsed with 1 μCi of tritiated methyl thymidine (^3H -TdR) for 5 hours, 37°C and then harvested onto glass filter paper using a semi-automatic cell harvester (Titertek cell harvester, Flow Laboratory) or a automatic cell harvester (TOMTEC, HARVESTER 96). ^3H -TdR incorporation was measured by a liquid scintillation counter (LKB-Wallac 1218 Rackbeta or Wallac 1450

Microbeta). The cultures were set up in triplicate for each antigen concentration and the data has been expressed as the mean (CPM) \pm standard deviation. Medium alone or final 5 μ g Con A/ml were used as negative and positive controls.

For antigen-specific T cell lines, $3-5 \times 10^4$ antigen-specific T cells were mixed with 2×10^5 irradiated autologous PBMC in 100 μ l RPMI / 10%FCS and antigen (100 μ l) and then cultured as previously described. Antigen-specific T-cells, or irradiated PBMC alone, antigen-specific T cells with antigen, or antigen-specific T cells with irradiated PBMC were set up as background controls with final 5 μ g Con A/ml as positive controls.

2.9.4 Antigen presenting ability of cultured MDM and AM

MDM were cultured, infected with MVV (1-2 TCID₅₀ per cell in 25 cm² flasks) and harvested as previously described. $1-2 \times 10^4$ viable MDM per well were plated into 96-well flat-bottom microtiter plates. Antigen-specific T cells ($3-5 \times 10^4$ cells per well) and antigen were added to a total volume of 200 μ l in RPMI / 10%FCS. The cells were cultured for 5 days at 37°C, 5% CO₂ and then ³H-TdR incorporation over the last 5 hours measured as section 2.9.3.

Sheep 1203T developed serious arthritis and was humanely euthanized during the course of these experiments. AM were collected from its lungs and used to test the antigen presenting ability of AM using cell concentrations as for MDM.

2.10 Cytotoxic Assays

2.10.1 Generation of virus-specific, cytotoxic T lymphocytes (CTL)

PBMC were obtained from MVV-infected sheep and placed into gelatin-plasma coated 75 cm² flasks. After 1 hour at 37°C incubation, non-adherent cells were collected as peripheral blood lymphocytes (PBL) which were then used to generate MVV-specific, cytotoxic T lymphocytes. The adherent cells were cultured as MDM and used as target cells in the cytotoxic assay. 2×10^6 PBL/ml of

complete RPMI / 10%FCS with 5 U rhIL-2 per ml were cultured with 10^5 autologous skin fibroblasts which had been infected with MVV, 0.5 TCID₅₀ per cell in DME / 2%FCS, for 1.5 - 2 hours. Cells were cultured in 1 ml per well of 24-well plates at 37°C, 5% CO₂ for 3-4 days and then fed with 0.5ml RPMI / 10%FCS with 5 U rhIL-2 per ml. After 7 days culture, the non-adherent cells were harvested and viable cells counted using trypan blue exclusion. One $\times 10^6$ viable cells per ml in RPMI / 10%FCS with 5 U rhIL-2 were seeded onto newly cultured, MVV-infected skin fibroblasts for 7 days as described above. After the end of the second cycle, cells were harvested, overlaid onto Lymphoprep, and centrifuged (750 xg, 10 minutes). Viable cells were harvested at the interface and washed twice with warm RPMI / 2%FCS. Cells were counted in 1% trypan blue and made to the required concentration in RPMI / 10%FCS for use as effectors in CTL assays.

Alveolar lymphocytes in BAL fluid were purified by depletion of adherent AM (2×10^7 BAL cells were incubated in 175 cm² flasks for 1 hour at 37°C). After two rounds of adherence, non-adherent cells contained around 80% lymphocytes and 20% AM. These cells were cultured in the same manner as PBL on autologous, MVV-infected skin fibroblasts with IL-2 and used as effectors in assays. The ability of AM to activate CTL *in vitro* was assessed by using AM to present live viral antigen to autologous PBL from MVV infected sheep. Two $\times 10^5$ AM per well were infected with MVV 1 TCID₅₀ per cell for 2 hours, 37°C. 2×10^6 PBL were added in RPMI / 10%FCS with IL-2 as above and incubated for 7 days, 37°C, 5% CO₂. Viable lymphocytes were transferred to AM which had been cultured for 7 days then infected as above, and the lymphocytes cultured for a further 7 days before harvesting as before.

2.10.2 Preparation of target cells

Both MDM (see section 2.12.1) and skin fibroblasts, either autologous or heterologous, were used as target cells. $1-2 \times 10^4$ MDM in complete

RPMI / 10%FCS or 1×10^4 skin fibroblasts in DME / 10%FCS per well were plated into 96-well flat-bottomed microtiter plates and incubated overnight at 37°C, 5% CO₂. Cells were either mock infected or infected with MVV, using 0.5 (skin fibroblasts) or 1-2 TCID₅₀ (MDM or AM) per cell, and incubated for 24 hours (skin fibroblasts) or 48 hours (MDM). Each well was labelled with 1.5 µCi (for Wallac micro-β counter) or 2.5 µCi (for LKB Wallac RIA Gamma counter) Na₂⁵¹CrO₄ in 50 µl medium (DME / 2%FCS or RPMI / 10%FCS, respectively) and incubated overnight at 37°C, 5% CO₂. The cultures were washed four times with RPMI / 2%FCS and then used as target cells.

AM were cultured in 25 cm² flasks and then mock-infected or infected with MVV, 1 TCID₅₀ per cell. AM in flasks were labelled overnight with 100 µCi Na₂⁵¹CrO₄ in 2 ml medium at 37°C, 5% CO₂. AM were harvested and washed four times with RPMI / 2%FCS and then used as target cells.

2.10.3 CTL-mediated cytotoxicity

Effector cells were made to the required cell concentration in RPMI / 10%FCS, usually 3 dilutions, eg 6×10^5 , 1.2×10^6 , 2.5×10^6 cells per ml to give effector : target ratios of 12:1, 25:1 and 50:1. 200 µl of the effector cells (CTL) were added to each well of washed targets. The plates were centrifuged (200xg, 1 minute) and incubated at 37°C, 5% CO₂. After 6 or 16 hours incubation, either 100µl of the supernatant was taken and counted in a gamma counter (LKB-Wallac 1274 Riagamma), or 40µl of the supernatant was mixed with 120µl Optiphase "Hisafe 3" (Wallac) in a 96-well Falcon flexible ELISA plate and counted by scintillation counting (Wallac 1450 Microbeta) for one minute. Assays were performed in triplicate for each dilution of effector cells. The minimum release was taken from wells without added effectors and maximum release was measured by addition of 1% Triton X-100 to the targets. Results were expressed as the percentage specific lysis, calculated as below:

specific lysis (%) = (mean cpm test - mean cpm minimum) / (mean cpm maximum - mean cpm minimum) x 100 \pm (standard derivation of cpm test/ mean cpm maximum-mean cpm minimum) x 100.

2.10.4 Depletion of lymphocytes with monoclonal antibody plus complement

Lymphocytes (1×10^7) were incubated for 40 minutes 0°C in 500 μ l of SBU-T4 (1 μ g/ml), or SBU-T8 (1 μ g/ml) in RPMI / 2%FCS. The cells were washed twice in RPMI / 2%FCS and then suspended in 500 μ l newborn rabbit serum (1:5 dilution in RPMI / 2%FCS) for 30 minutes at 37°C. After one wash with RPMI / 2%FCS, the complement depletion was repeated once more. The cells were washed twice with RPMI / 2%FCS and then suspended in RPMI / 10%FCS. Viable cells were counted in 1% trypan blue and diluted to the required cell concentration for effectors in CTL assays.

Depletions were checked by antibody staining and analysis on the FACS using FACSCAN software. Dead cells were gated out by staining with 20 μ g/ml propidium iodide in RPMI / 2%FCS after monoclonal antibody staining.

2.10.5 Cytotoxic activity of lymphokine-activated killer cells (LAK)

PBMC were prepared as described in section 2.2. The adherent cells (monocyte-enriched cells) were depleted and cultured as target cells (section 2.10.2). The PBL (non-adherent cells) were cultured in 25cm² flasks at a concentration of 3×10^6 cells/ml RPMI / 10%FCS in the presence of 10U rhIL-2/ml for 5 days or 13 days. More rhIL-2 (10U) was added on culture day 3. The effector cells were overlaid onto Lymphoprep and viable cells harvested from the interface (500 xg, 10 minutes). After 2 washes with RPMI / 2%FCS, the viable cells were counted and diluted to the required concentration in RPMI / 10%FCS.

To separate FcR⁺ and FcR⁻ cell populations, 2×10^6 PBL/ml were mixed with 50 μ l 1% opsonized sheep RBC (section 2.8.3) and incubated at 37°C for 10-

15 minutes. The cell mixture was centrifuged (200 $\times g$ for 3 minutes), resuspended in PBS and incubated for another 30 minutes at 37°C, 5% CO₂. The cell mixture was diluted and overlaid onto Lymphoprep. After centrifugation (500 $\times g$, 10 minutes), erythrocyte rosette positive (ER⁺) cells were at the bottom of the tube, and erythrocyte rosette negative (ER⁻) cells were at the interface. Both populations were collected separately, diluted in RPMI / 2%FCS and washed twice. ER⁺ population were lysed with buffer (10 mM potassium bicarbonate containing 0.155 M ammonium chloride and 0.1 mM EDTA) and washed twice in RPMI / 2%FCS. Both ER⁺ and ER⁻ population were cultured in 25 cm² flasks as described above. These lymphokine activated killer cells were then used as effectors in cytotoxicity assays as described in section 2.10.3.

The target cells, MDM and skin fibroblasts, either autologous or heterologous, were prepared as previously described (section 2.10.2).

2.11 Experimental Sheep

Finnish Landrace crossed sheep (1-4 years old), were purchased from the Moredun Research Institute, Edinburgh. Uninfected sheep used for the generation of antigen-specific T cell lines were kept at the Moredun Research Institute. MVV-infected sheep were kept at the Bush Field Station, Easter Bush Estate, Edinburgh. In addition, lungs for normal BAL were taken from abattoir material or experimentally culled sheep at the Moredun Research Institute. They were of mixed breed and age (adult).

Sheep naturally-infected sheep with MVV from a contaminated flock (specimens kindly given by Dr. N. J. Watt) were also used. These were mostly adult Texel crosses.

CHAPTER 3

ULTRASTRUCTURAL OBSERVATION OF MAEDI VISNA VIRUS REPLICATION IN MACROPHAGES AND SKIN FIBROBLASTS

3.1 Introduction

In vivo, MVV replication in monocytes and macrophages is restricted so that only small amounts of viral protein are detected, although many copies of viral RNA may be seen in target cells (Gendelman et al., 1985). There is also no evidence of cell free viremia (Petursson et al., 1976; Bird et al., 1993), therefore it is unlikely that large numbers of virions are produced *in vitro*. The restriction of virus replication may be due to the infected cell type (macrophages), maturation stage of monocyte/macrophages, LV-IFN as well as other cytokines and host immune surveillance (Narayan and Clements, 1989). *In vitro* MVV replication is productive in both macrophages and fibroblasts. MVV replication in sheep choroid plexus (SCP), or skin fibroblast cells induces cytopathic effects (CPE) which are characterized by the formation of syncytia and cellular lysis which coincides with the period of peak virus production (Thormar, 1961; Macintyre et al., 1973). However, the CPE seen in MVV-infected cultures is viral isolate and cell type dependent (Narayan et al., 1982; Lairmore et al., 1987).

Virus isolates have been divided into three different groups by their replication patterns in sheep fibroblasts and macrophages (Lairmore et al., 1987). Group replication patterns are (1): growth to high virus titer and many syncytia, (2) high titer but few syncytia, or (3) low titer and no syncytia in both macrophages and synovial cells. These different groupings correspond to pathogenicity when isolates are used to experimentally infect lambs (Lairmore et al., 1987). In addition, phenotypically distinct MVV isolates induce different immune responses to *env* proteins compared to *gag* proteins (Kajikawa et al., 1990). The rapid/high virus-infected group developed a five-fold higher geometric mean titer of anti-*env* antibody than did the slow/low virus infected group *in vivo* (Kajikawa et al., 1990).

To date sheep choroid plexus (SCP) culture has been widely used in the study of MVV. Using transmission electron microscopy (TEM) many viral budding sites have been found at the cytoplasmic membrane of SCP (Thormar, 1961;

Macintyre et al., 1973; Narayan, et al., 1982) as is the case for CD4⁺ T cells in HIV replication (Palmer et al., 1985; Meltzer, et al., 1990). In contrast, only a few viral budding sites are present at cytoplasmic membranes of HIV infected macrophages (Orenstein et al., 1988) or MVV-infected macrophages (Narayan et al., 1982). Most HIV and MVV virions in macrophages accumulate in vacuoles. *In vitro*, MVV replication in skin fibroblasts or choroid plexus cells has been extensively studied, but MVV replication in macrophages, especially by electron microscopy, is still limited.

HIV replication has been compared in lymphocytes and macrophages. More than 35% of total viral proteins synthesized are *env* gene products in HIV-infected lymphocytes. In contrast, a relative lack of *env* protein, not more than 10% of total viral protein, was seen in HIV-infected macrophages (reviewed in Meltzer et al., 1990). This data suggests that HIV derived from macrophages is relatively deficient in *env* protein and so may be less infectious than HIV derived from lymphocytes through CD4 entry or may use other virus receptor molecules. Whether MVV synthesis and assembly in macrophages is similar to that seen in HIV infected macrophages is not documented.

A British isolate of MVV (strain EV1) was isolated by cocultivation of peripheral blood mononuclear cells of a sheep with an acute maedi-like disease with ovine skin fibroblasts (Sargan et al., 1991). This strain shows typical CPE (syncytia and lytic infection in skin fibroblasts). However, EV1 replication in monocytes and macrophages has not been studied. Therefore, the characteristics of EV1 replication and viral protein synthesis in monocytes and macrophages were studied and compared to those in skin fibroblasts.

3.2 Results

3.2.1 Maedi-visna virus (strain EV1) replication in monocytes and AM

During EV1 replication in MDM (infection on day 7), there were no remarkable morphological changes in the first 4 days post infection under light microscopic examination, compared with uninfected controls. But 5 days post infection some infected cells became small and refractive and gradually lysed in the culture. Compared to uninfected monocytes or macrophages, about 30-40% of cells were lost within the first 6 days. By day 9, about 60% to 80% of cells were detached from the well.

The time course of MVV replication in monocytes and macrophages is shown in figure 3.1. No virus was detected in the supernatant of fresh monocytes infected with virus by day 3 post infection. However, virus production was seen by day 4 which peaked at day 7 post infection. In contrast to fresh monocytes, 7 day-cultured MDM infected with MVV showed 10^4 - 10^5 TCID₅₀/ml in the supernatant by day 2 post infection with peak titers at day 4-8 post infection. Three-day cultured monocytes infected with MVV showed intermediate viral production in the supernatant compared to fresh monocytes and day 7 cultured monocytes. In contrast, MVV replication in AM showed a steady increase in the virus titer in the supernatant with time. At the peak of virus production, viral infected monocytes, MDM and AM had degenerated and eventually lysed in the culture. The typical fibroblast CPE, syncytia, were not evident in infected fresh and 3-day cultured monocytes. Syncytia were seen in 7-day cultured MDM infected with MVV. These syncytia (often 4-8 nuclei) were not as large as those seen in skin fibroblasts (often more than 8 nuclei) infected with MVV. For the remaining experiments (TEM and SEM observation) 7 day cultured MDM were always used for infection.

Fluorescence antibody (FA) staining of virus-infected MDM for *gag* protein, p15, also showed increasing amounts of this protein with time. On day 2 post infection, 20 to 30% of MDM showed a weak positive specific fluorescence for p15

Figure 3.1 Maedi-visna virus (EV1) replication in monocytes, MDM and AM

2.5×10^6 PBMC (for MDM) or 2.5×10^5 AM were seeded in 24-well plates and nonadherent cells were removed as described in materials and methods (section 2.2.3 & 2.2.4). Monocyte/MDM were infected with EV1 at 1 TCID₅₀ per cell on day 0, 3 or 7 and AM were infected on culture day 1. After 2 hours incubation, non-absorbed viruses were removed and cultures were re-fed with fresh medium. Supernatant was collected every two days and virus titers were assessed on skin fibroblasts as described in section 2.3.2.

FIGURE 3.1

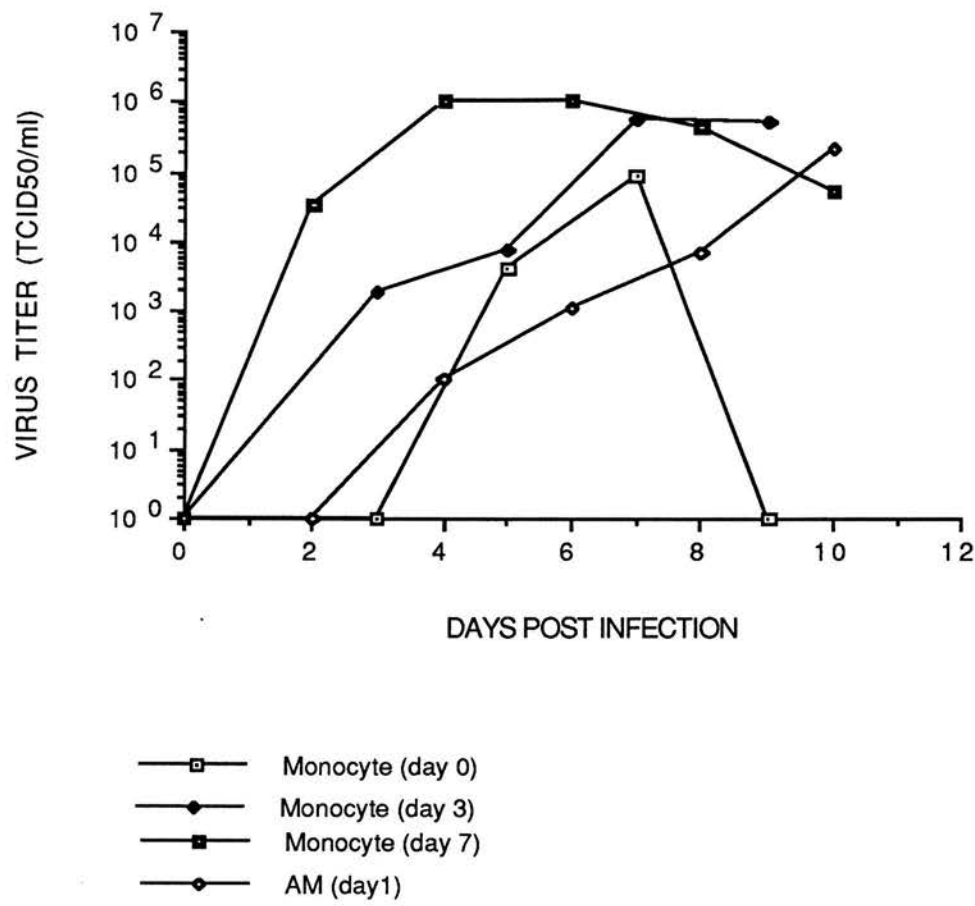


Figure 3.2 Antibody staining of MDM after infection with MVV

MVV-infected and mock-infected MDM (infection on day 7) were fixed in methanol. Cells were stained with 415 monoclonal antibody to viral *gag* protein, p15, followed by anti-mouse IgG-biotin and streptavidin as described in section 2.4.1. Mock-infected MDM stained with 415 monoclonal antibody were used as negative controls.

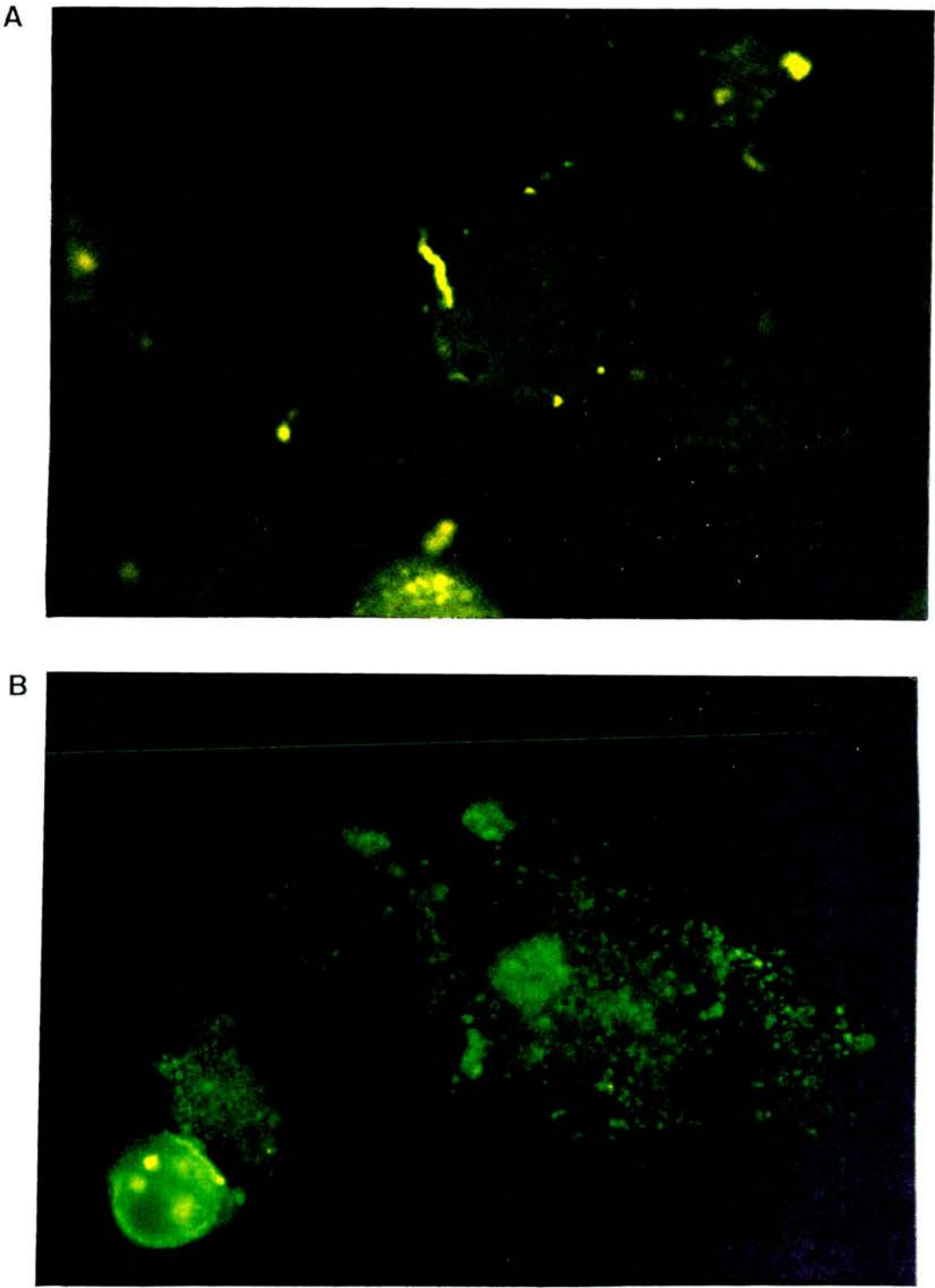
A. Mock infected MDM.

Magnification: x1,000

B. MVV-infected MDM 7 days post infection.

Magnification: x1,000

FIGURE 3.2



antigen in their cytoplasm, but after 4 days post infection, over 80-90 % of MDM were strongly positive for p15 antigen (figure 3.2.B).

3.2.2 Culture of monocytes from MVV-infected sheep

Monocytes cultured from MVV-infected sheep often gave poor cell recoveries when compared to those from uninfected sheep. Therefore, cultured monocytes from normal or MVV-infected sheep, and detection of viral antigen by FA staining on cultured MDM were investigated and compared. Monocytes from normal sheep gradually increased their size and cytoplasm : nucleus ratio in the first 7-10 days and cultures became nearly confluent (figure 3.3.A). Thereafter, macrophage proliferation in the culture could be found. Monocytes from MVV-infected sheep, especially naturally or long-term experimentally infected sheep, had apparently normal monocytes for the first three days in culture but the cell density was less than that from normal sheep. More cells became detached from the plastic during culture, especially macrophages from naturally infected sheep. There was also great variation in the CPE seen in monocytes between MVV-infected sheep. Syncytia (figure 3.3.B) could be found in cultured monocytes from some sheep (4 out of 7 naturally infected-sheep) after 7 days culture, but were not noticeable in monocytes from experimentally infected sheep. Cultures containing syncytia were also positive (low frequency) for viral p15 *gag* antigen (data not shown). Furthermore, cultured MDM from one naturally infected sheep which showed syncytia formation were processed for TEM observation and MVV particles could be seen in the cytoplasm of the cells (data not shown).

3.2.3 TEM observation of MVV (EV1) infected MDM

By day 4 post infection with MVV, MDM showed no gross changes in cellular microorganelles, except for an increase in the amount of endoplasmic

Figure 3.3 Cultured MDM from MVV-infected sheep

MDM from uninfected and MVV-infected sheep were cultured in gelatin-coated 24-well plates as described in section 2.2.3. After 7 days culture, MDM were fixed in methanol and then stained with Giemsa's stain.

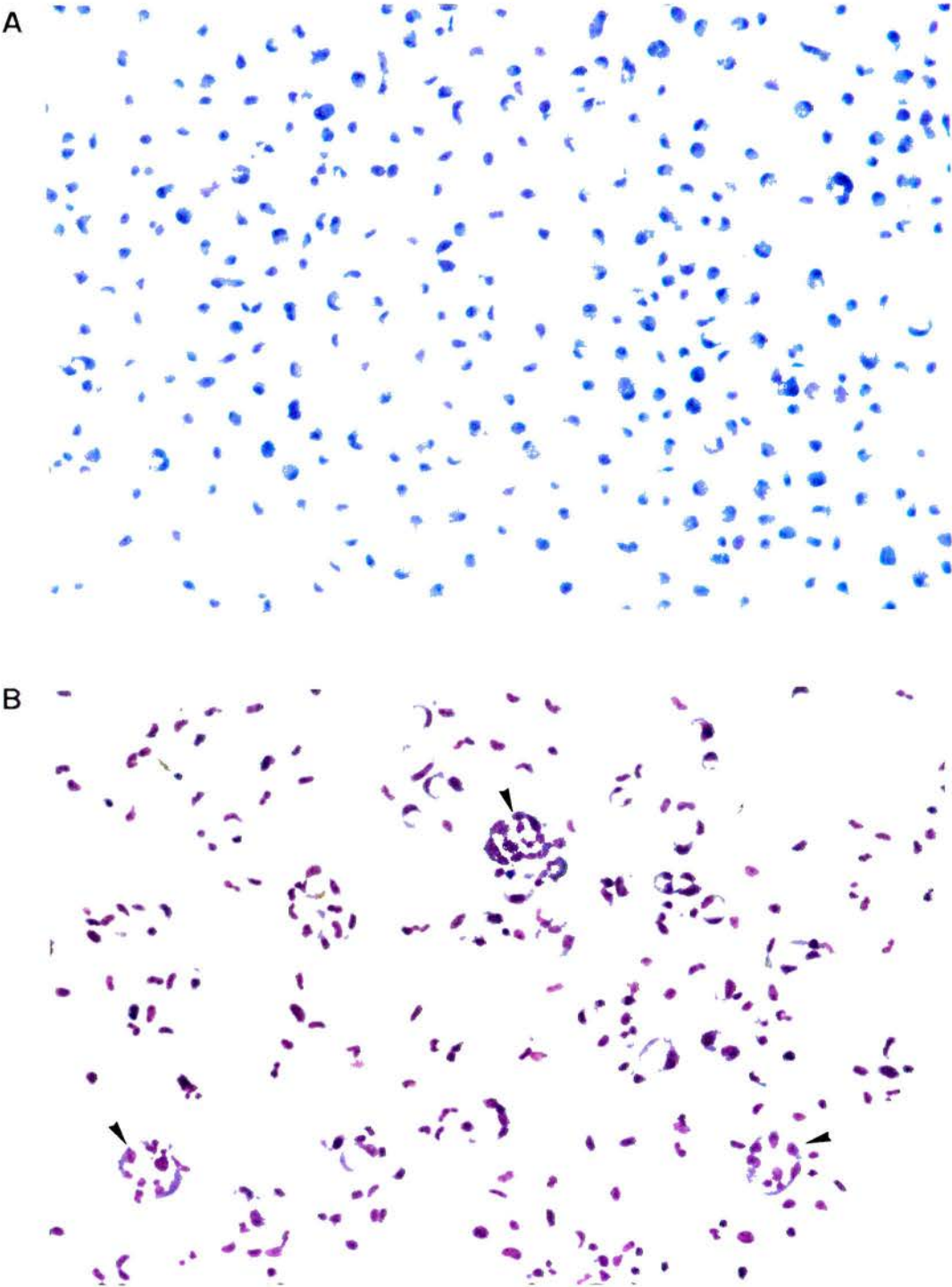
A. MDM cultured from an uninfected control sheep.

Magnification: x200

B. MDM cultured from a naturally MVV-infected sheep which had pulmonary lesions. Note the characteristic syncytial cells (➤) present in the culture.

Magnification: x200

FIGURE 3.3



reticulum and number of ribosomes. The morphological changes seen in infected MDM by 6 to 9 days post infection were variable in each cell, including profound vacuolation of the cytoplasm and swelling of mitochondria and the nuclei contained dispersed heterochromatin margins (figure 3.4 b & c). Some MVV-infected MDM lost their surface processes. However, there were no obvious syncytia found in these sections. Virus budding was seen from both the cytoplasmic and vesicular membranes of the cells and virions were seen accumulating both in cytoplasmic vacuoles and in intercellular spaces.

There were two types of viral particles seen in MVV-infected MDM. The first form was intracytoplasmic particles (figure 3.4.f), which had a similar morphology to intracytoplasmic type A (ICA) particles of the type B and D retroviruses (Fine and Schochetman; 1978). The ICA particles were about 80-100nm in diameter, round in shape with one electron dense incomplete or complete ring, and electron-lucent centers. A ring of ribosome-like substance surrounded the particle surface. Sometimes, the aberrant forms with 2 or 3 electron-dense rings were seen. The ICA particles were easily seen in the cytoplasmic matrix, some of them being closely associated with the endoplasmic reticulum. No ICA particles were found within vesicles although they were often seen around the vesicles (figure 3.4 e). Moreover, no ICA particles were seen budding from the cytoplasmic membrane in these specimens.

The second type of viral particle was mature or immature virions which had electron-dense, round or bar-like nucleoids and were about 70-120nm in diameter (figure 3.5 c). The shape and size depended on the plane of section and the maturity of the virion. Mature viral particles were smaller than ICA particles and were about 70 to 90 nm in diameter. Most mature virions had a round to bar-like nucleoid (occasional coffin shaped nucleoids were seen) which was centrally placed in the virion, but sometimes, two or more round nucleoids were observed. In contrast, immature virions which had just been released from the cytoplasmic membrane

were bigger in size, 90 - 120nm, and had a circular electron-dense nucleoid (figure 3.5.c). Mature viral particles predominantly accumulated in vesicular lumens (figure 3.5.b) and extracellular areas (figure 3.5.a).

During viral budding, an electron-dense nucleoid formed with a crescent shape which fused with the vesicular (figure 3.4.f) or cytoplasmic membrane (figure 3.5.a). Both ends of the crescent eventually met and then formed a ring-like nucleoid with an electron translucent center. After that, virions were released from the membrane as immature virions. These immature virions had a ring shaped nucleoid and very short, faint spikes (figure 3.5 a). The ring-like nucleoid gradually condensed and became centrally located as in the mature virions. Between 5 and 7% of viral containing MDM on day 8 and 9 post infection showed viral budding sites on their cytoplasmic membrane. Compared to the number of ICA and mature particles which had accumulated, there were only a few viral budding sites present at the vesicular and cytoplasmic membranes (TEM only sees a small percentage of membrane surface area). However, a lot of viral buds were found at the cytoplasmic membrane in a few MDM, especially smaller MDM (figure 3.4.c & d). Clustered viral particles found in the extracellular spaces (figure 3.4.d) might have been released from cells by exocytosis or cellular lysis.

3.2.4 TEM observation of MVV (EV1) infection in AM

EV1 replication in AM was very similar to that found in MDM. There were more ICA particles (figure 3.5.e&f) found in the cytoplasmic matrix and fewer mature viral particles accumulated in vesicles (figure 3.5.d). It was very difficult to find viral budding at the cytoplasmic membrane, and there were only a few viral buds found on vesicular membranes in these specimens. In a time course of infection, only a few virions could be found in AM by day 4 post infection, whereas, nearly 70% of AM on 9 days post infection contained ICA or mature viral particles (data not shown).

Figure 3.4 Transmission electron micrographs of MVV-infected MDM

MDM were cultured in 75cm² flasks (day 7) and then infected with EV1, 1 TCID₅₀ per cell. After 6-7 days incubation, MDM were pelleted and fixed in 3% glutaraldehyde in cacodylate buffer and then post-fixed in osmium tetroxide. Ultrathin sections were made and double stained with uranyl acetate and lead citrate and then observed under a Philips 300 TEM.

a. Uninfected MDM showed intact processes, microorganelles and nucleus.

Magnification: x7,000

b. MVV-infected MDM 6 days post infection. Both virus-infected cells had become very vacuolated. Magnification: x3,750.

c. MVV-infected MDM 6 days post infection. A lot of viruses were budding from the cytoplasmic membrane (➤). Magnification: x7,700.

d. MVV-infected MDM 7 days post infection. The small MDM in the right hand corner showed a few viral budding sites (➤) at its cytoplasmic membrane. In contrast, well differentiated MDM became very vacuolated (upper right). Clumps of viral particles (➡) accumulated in intercellular spaces. Magnification: x7,700.

e. MVV-infected MDM 7 days post infection. A few viral particles were present in the vesicular lumen and intracytoplasmic matrix. Viral budding sites were also present at the vesicular membrane (➤). High magnification of vacuole (V) is shown in photograph (f). Magnification: x10,000.

f. Viral buds on a vesicular membrane (➤) and intracytoplasmic type A (ICA) particles (➡). Magnification: x35,000.

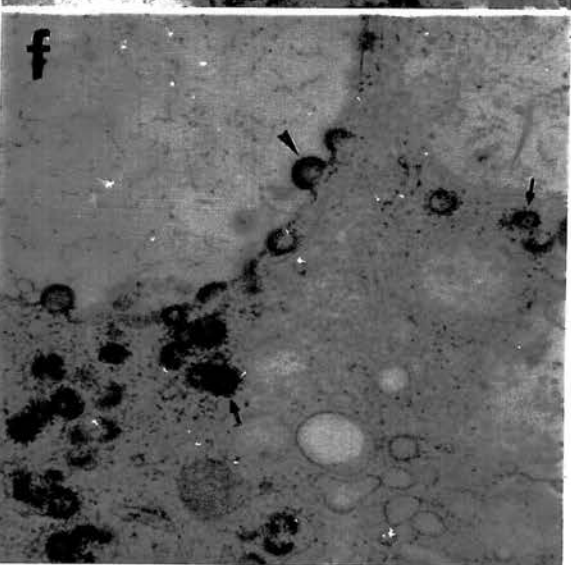
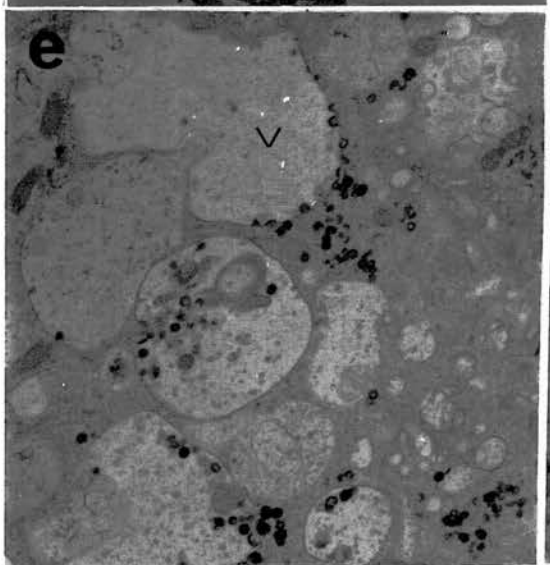
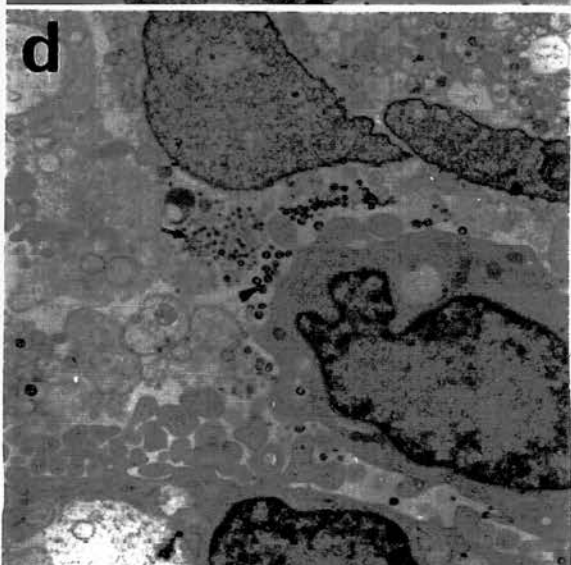
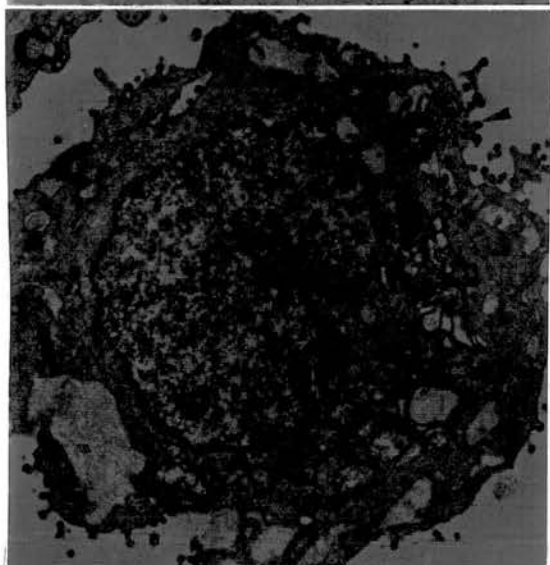
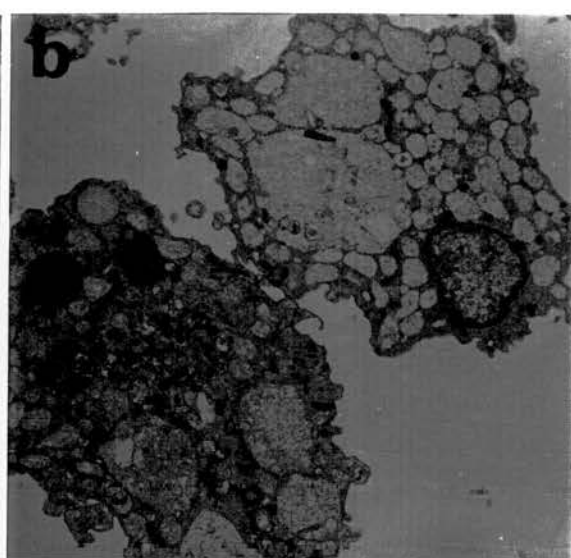
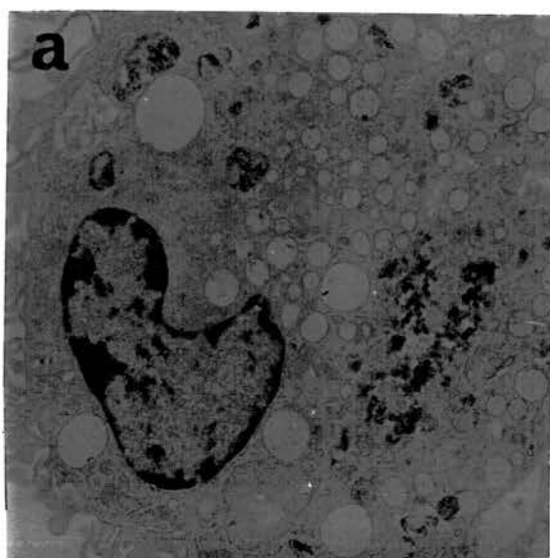


Figure 3.5 Transmission electron micrographs of MVV-infected MDM (a- c) and AM (d-f)

The MDM and AM specimens were processed as prescribed in section 2.5.

a. High magnification of figure 3.4d. Viral budding sites at the cytoplasmic membrane (➤) and virions in the intercellular space (→).

Magnification: x46,000.

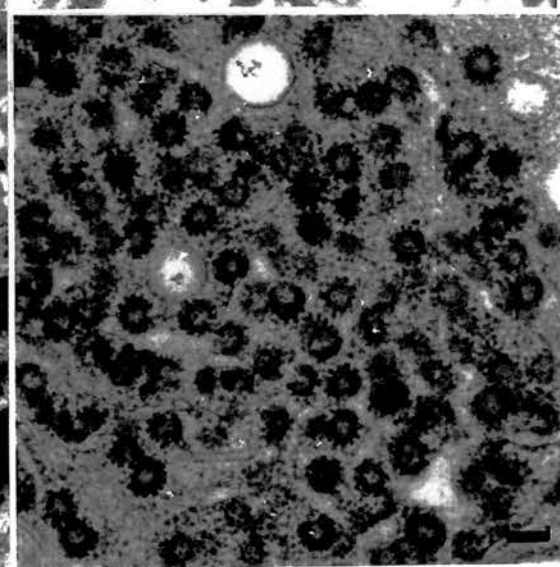
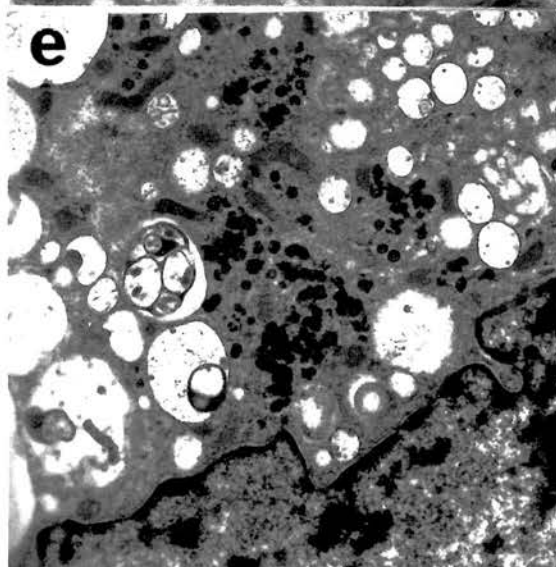
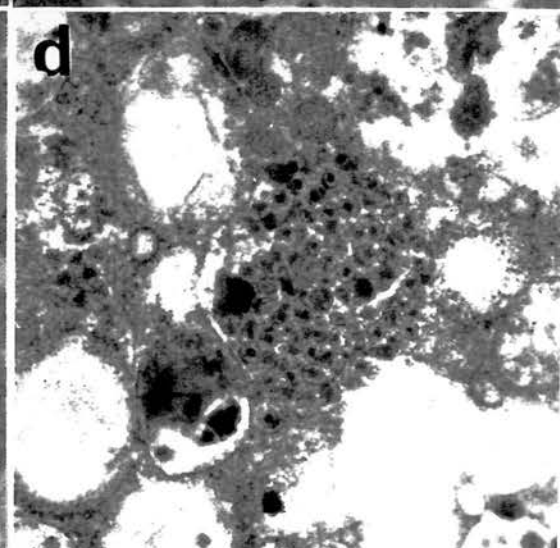
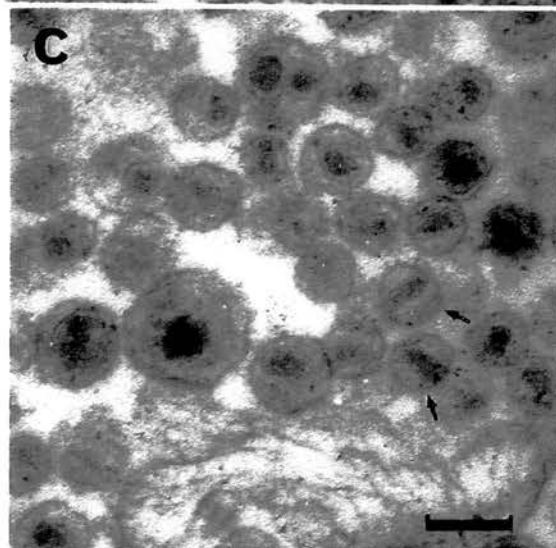
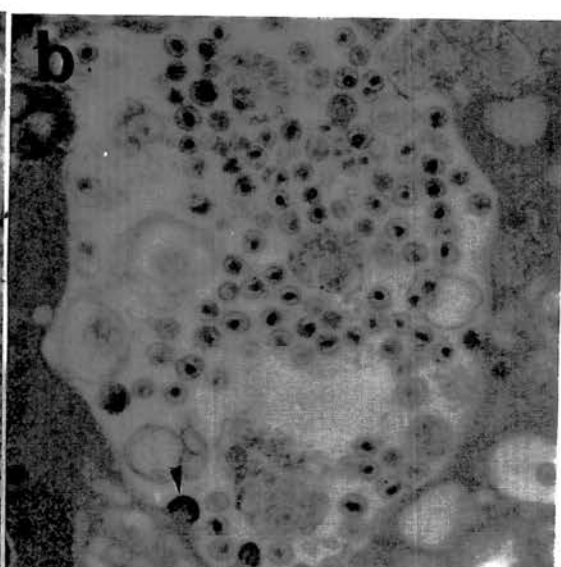
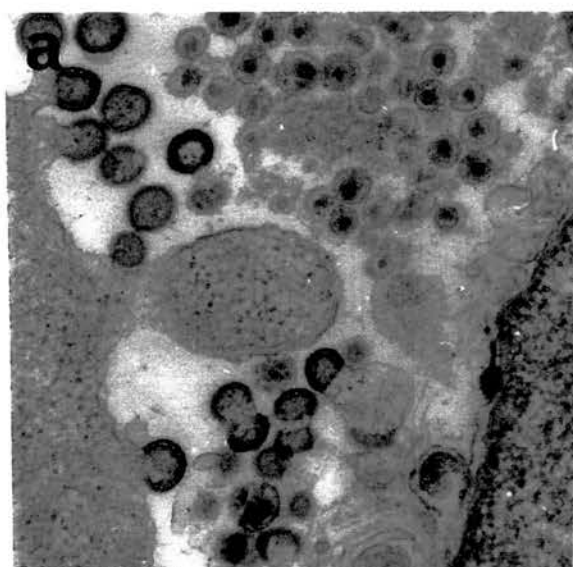
b. MVV-infected MDM 7 days post infection. A large number of viral particles accumulated in the vesicular lumen and viral budding was present at the vesicular membrane (➤). Magnification: x37,500.

c. MVV-infected MDM 7 days post infection. Mature virions (→) contain a bar-like nucleoid and have a size of 70-90 nm in diameter. In contrast, immature virions (➤) have a denser central nucleoid and are larger than mature virions. Space bar = 100nm. Magnification: x60,000.

d. MVV-infected AM 8 days post infection. A clump of viral particles accumulated in a vacuole. Magnification: x35,500.

e. MVV-infected AM 8 days post infection. Viral particles (ICA) scattered in the cytoplasmic matrix. Magnification: x12,500.

f. MVV-infected AM 8 days post infection. Typical ICA particles in the cytoplasm. Space bar = 100nm. Magnification: x46,000.



3.2.5 TEM observation of MVV (EV1) infection in skin fibroblast cells

In contrast to MVV replication in MDM, there were a lot of viral budding sites present at the cytoplasmic membrane of MVV-infected skin fibroblasts (figure 3.6.b) and numerous free viral particles were scattered in extracellular areas (figure 3.6.c). But only a few ICA viral particles were found in the cytoplasm of some infected skin fibroblasts at 8-9 days post infection (data not shown). Viral particles which had accumulated in the vesicles of MVV-infected skin cells were rare. Other morphological changes of MVV-infected skin fibroblasts included multiple nuclei (syncytia) (figure 3.6.a) and degeneration of mitochondria and nuclei.

3.2.6 Comparison of strain EV1 and 1514 replication in MDM

ICA particle formation is characteristic of type B murine and type D retroviruses (Spumavirus) (Fine and Schochetman, 1978) but not lentiviruses. The detection of ICA particles in MDM and AM could therefore be due to endogenous virus in these cell types which are activated by MVV infection. To address this question the Icelandic MVV strain, 1514, was used to compare with EV1. A single source of MDM was infected with EV1 and 1514 virus at the same time, cultured under the same conditions, and then processed for EM examination. MDM infected with EV1 contained ICA particles, mature and immature virions and viral budding sites similar to previous observations (figure 3.7. a, c, e). In contrast, 1514 virus-infected MDM did not contained ICA particles. Interestingly, 1514 viral budding sites found at the cytoplasmic membrane showed crescent nucleoids which invaginated and apparently released virions into the cytoplasm (figure 3.7. b, d, f). This phenomenon was different to EV1 which budded and released into the extracellular area (figure 3.7.c). Other ultrastructural changes in MDM infected with 1514 virus included serious hydropic degenerative changes in the cytoplasm, swelling of nuclei and dilation of vesicles.

Figure 3.6 Transmission electron micrographs of MVV-infected skin fibroblasts

Skin fibroblasts were cultured in T-75 flasks and then infected with MVV, 0.5 TCID₅₀ per cell. Depending on CPE formation, skin fibroblasts were harvested and processed as described in section 2.5.

a. MVV-infected skin fibroblasts 6 days post infection. The skin cell showed 3 nuclei and is probably a syncytium.

Magnification: x4,600.

b. MVV-infected skin fibroblasts 6 days post infection. A large number of viral buds were present at the cytoplasmic membrane.

Magnification: x 16,500

c. High magnification of viral budding sites (➤), immature viral particles (➡) and mature virions (➞). Space bar = 100nm.

Magnification: x60,000.

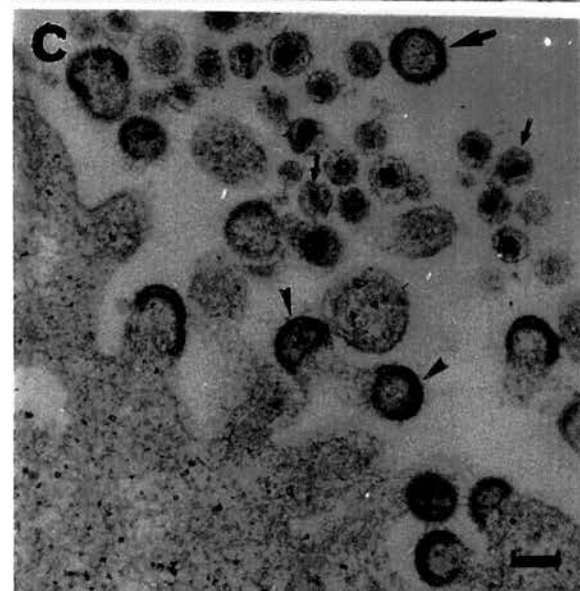
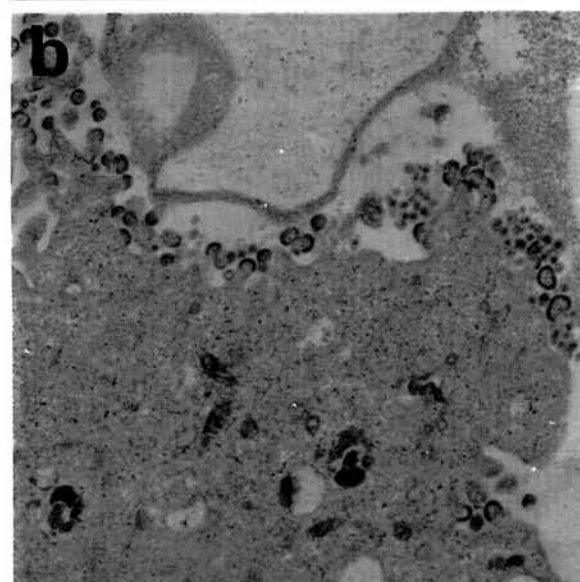
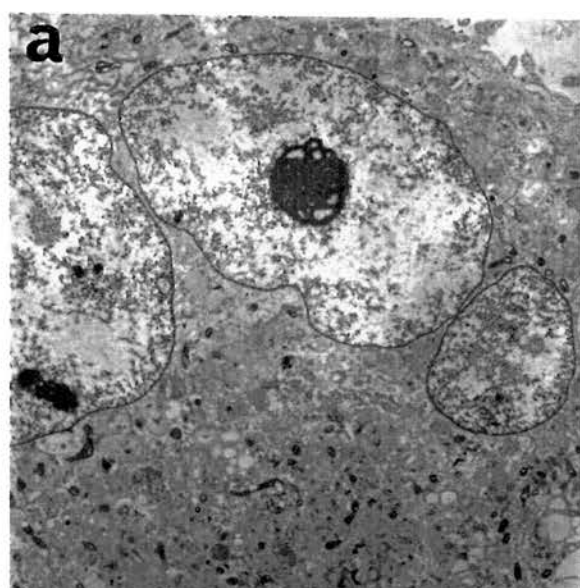


Figure 3.7 Comparison of strain EV1 (a, c, and e) and 1514 (b, d, and f) infected MDM.

MDM from the same source were infected with EV1 (a, c, e) or 1514 (b, d, f) virus , 1 TCID₅₀ per cell , respectively, and cultured under similar conditions. On 6 days post infection, cells were processed for TEM observation as previously described in section 2.5.

Both viral budding at the cytoplasmic membrane (➤) (box 1) and ICA particles in the cytoplasmic matrix (box 2) were found in the same cell infected with EV1. A higher magnification of viral budding sites and ICA particles in boxes in (a) are shown in (c) and (e) respectively.

Magnification:a: x10,000

c: x27,500

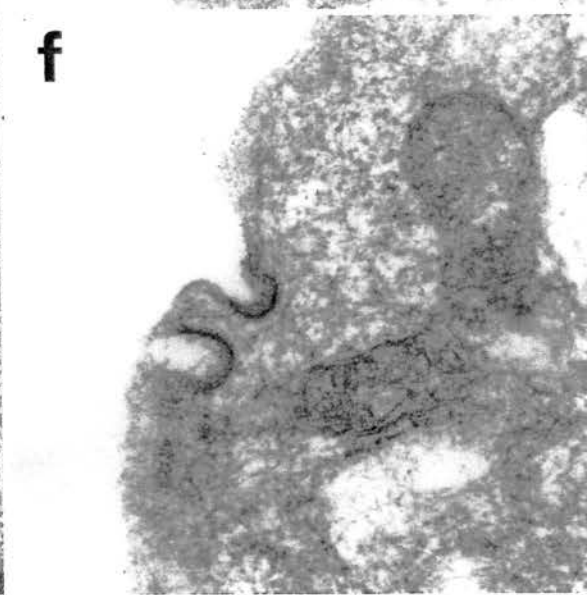
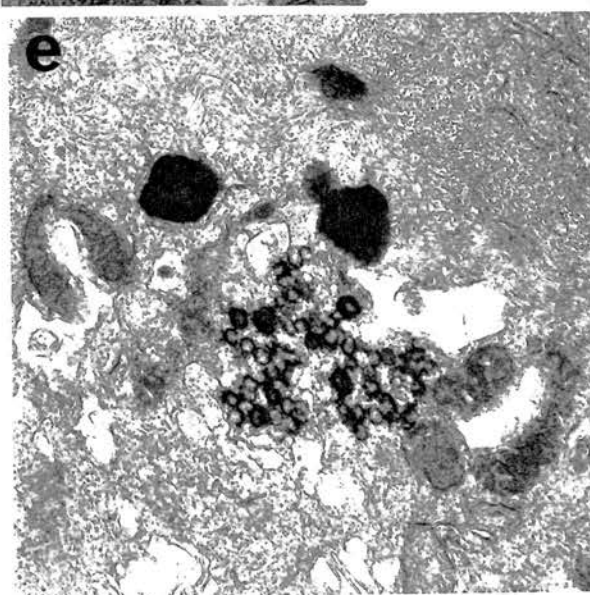
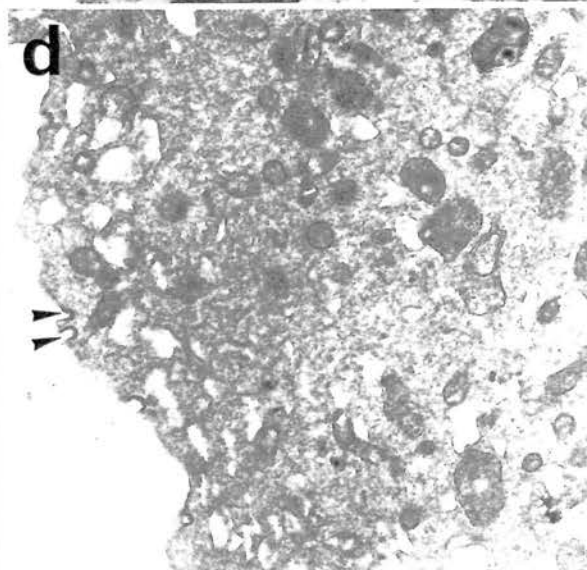
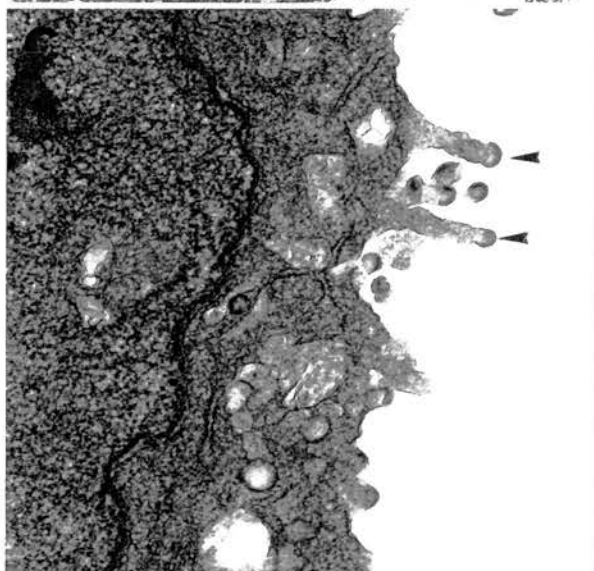
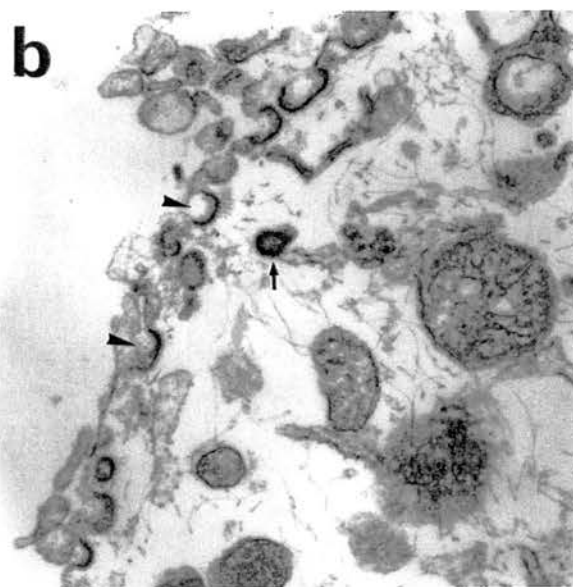
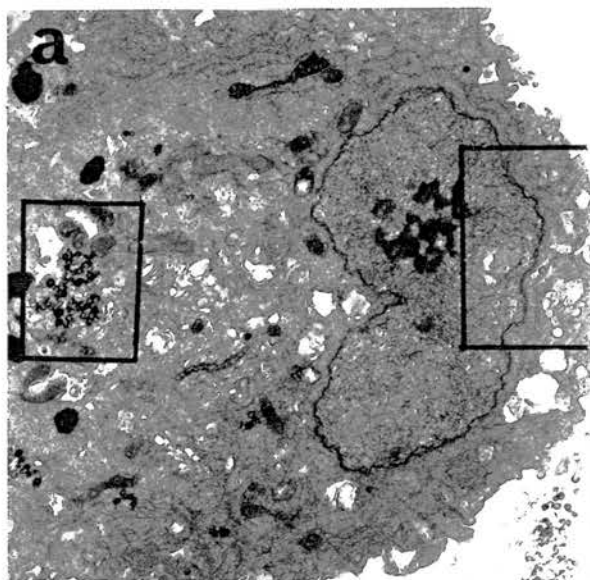
e: x27,500

Viral budding sites which appeared to invaginate into the cytoplasm (➤) were found in MDM infected with 1514 virus. An immature virion (➡) is shown in the cytoplasm (b). A higher magnification of the viral budding sites in (d) is shown in (f).

Magnification:b: x35,500

d: x12,500

f: x 60,000



3.2.7 Surface changes of MVV-infected skin fibroblasts and macrophages

Scanning electron micrographs of MVV-infected MDM were prepared 2, 4, 6, and 8 days post infection. The morphology of adherent MDM observed under SEM could be classified as three types. The first category was healthy or normal MDM which were well spread on the coverslips, with an abundance of membrane ruffles (figure 3.8.c). The second type of MDM had degenerated which was characterized by atrophy or partial loss of the membrane ruffles (figure 3.8.d) and decreased extension on the coverslip (figure 3.8.b). Moreover, these cells had surface globular structures which varied in size (0.5-1 μ m) and shape. These may be derived from the degenerating membrane ruffles or be viral budding sites. The third category of infected MDM had uniform and tiny globular structures (200-250nm) on their surface (figure 3.8.e, f). The severely degenerated MDM, predominantly found in the late phase of viral infection, had completely lost their membrane ruffles and pseudopods (data not shown).

In non-infected MDM, about 90% of cells were healthy with less than 10% having degenerated. By 2 days post infection, most MVV-infected MDM had similar morphology to non-infected MDM, but a few MDM (about 8%) showed a few globular structures on their surface. By 4 days post infection, the healthy, degenerate and severely degenerate cells were 80%, 15%, and 5%, respectively. Moreover, 36% of MDM exhibited globular structures on their surface membrane. By 6 days post infection, 35% of adherent MDM had degenerated and over 50% of them exhibited globular structures. After 8 days post infection, 70-80% of MDM were detached from the coverslip (figure 3.8.b) and over 90% of the remaining adherent MDM were degenerative or severely degenerative and showed variable globular structures on their surface. These results are summarized in table 3.1.

The surface changes on MVV-infected AM were different to those seen on MDM. It was difficult to find virion-like particles on the cell surface like those found on MDM. But in the early phase of viral infection, localized areas of surface

ruffles were lost and replaced by a bulge containing some small globular structures (figure 3.9.a, b), similar to those found on MDM. This phenomenon may be exocytosis of a virion-containing vesicle into the extracellular space. With increasing time post infection, surface ruffles were gradually lost and replaced by many such bulges and volcano-like structures (data not shown).

The change in surface morphology of skin fibroblasts infected with MVV was more easily observed than changes of MDM. Normal uninfected skin fibroblasts were very flat and well attached to the coverslip by extended filaments (figure 3.10.a). Their surfaces were very smooth and had a low contrast under SEM observation, but sometimes, a few globular structures varying in size and shape could be found on cells, which might be cell debris. However, with time after MVV infection, the number of degenerative cells increased in culture. They were characterized by shrinking of cellular filaments, and appeared to have a rough cell surface (figure 3.10.b). There were many uniform globular structures on the cell surface (figure 3.10.c). The globular structures were 200-250nm in diameter (figure 3.10.d). Some semi-globular structures with a similar size were seen to be just budding from the cell membrane and were probably sites of viral budding (figure 3.10.d). The proportion of cells positive for globular units on days 0, 2, 4, and 6 days post infection were 6%, 38%, 82% and 93%, respectively.

3.2.8 Detection of viral proteins synthesized in AM and skin fibroblasts

The difference in viral replication and assembly patterns found in AM and skin fibroblasts was very similar to that reported for HIV replication in macrophages and lymphocytes (Orenstein, et al., 1988; Folks et al., 1988; Gendelman et al., 1989), respectively. As there is a known difference in the amount of *gag* : *env* synthesized in macrophages and lymphocytes in HIV, the synthesis of *gag* and *env* was studied in MVV infected macrophages and skin fibroblasts.

Figure 3.8 Scanning electron micrographs of MVV-infected MDM

MDM were cultured on coverslips in 24-well plates. On day 7 MDM were infected with MVV, 1 TCID₅₀ per cell. At designated times cells were washed with PBS 5-6 times, then fixed in 3% glutaraldehyde in cacodylate buffer at 4°C overnight and post-fixed in osmium tetroxide for 1 hour. The cells on coverslips were dehydrated in graded acetone and then dried in a CO₂ critical point dryer. Cell surfaces were coated with evaporated gold and observed under SEM.

- a. Uninfected MDM were confluent in culture. Magnification: x120.
- b. MVV-infected MDM 8 days post infection. Most virus infected cells had lysed and were detached from the coverslip. Magnification: x625
- c. Uninfected MDM had intact membrane ruffles and was evenly spread on the coverslip. Magnification: x3,100
- d. MVV-infected MDM 6 days post infection. The degenerative MDM showed partial loss of membrane ruffles and there were a lot of globular structures of various sizes on its surface. Magnification: x5,000
- e. MVV-infected MDM 6 days post infection. Similar to (d), but the cell surface was covered by more uniform globular structures which may be viral particles.
Magnification: x2,500
- f. High magnification of viral particles of (e). Space bar = 1µm.
Magnification: x10,000.

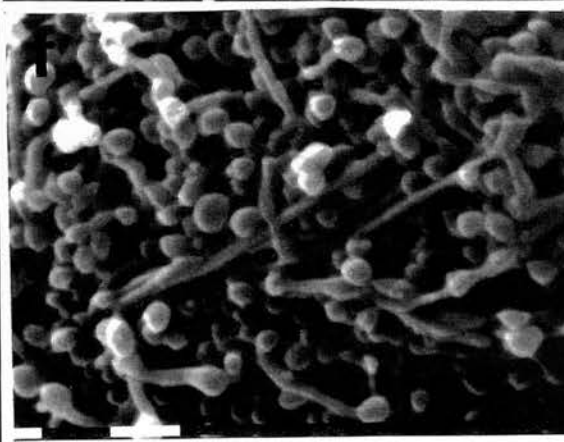
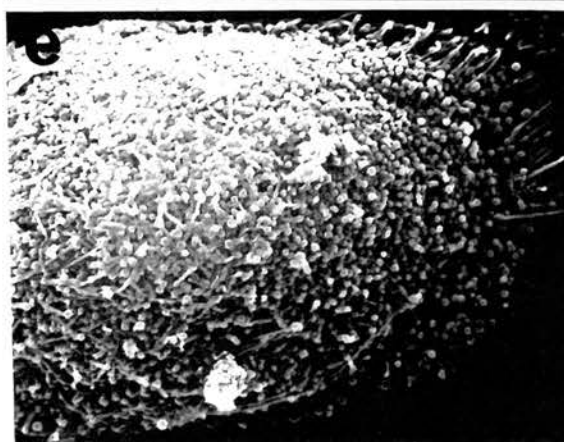
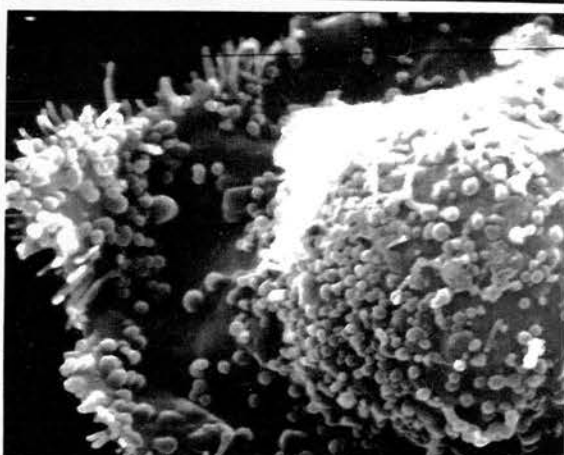
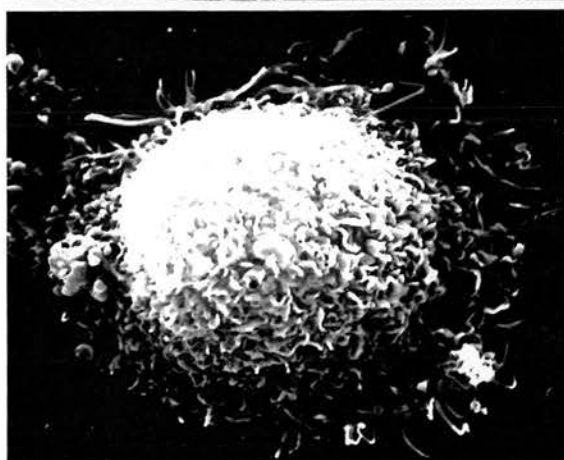
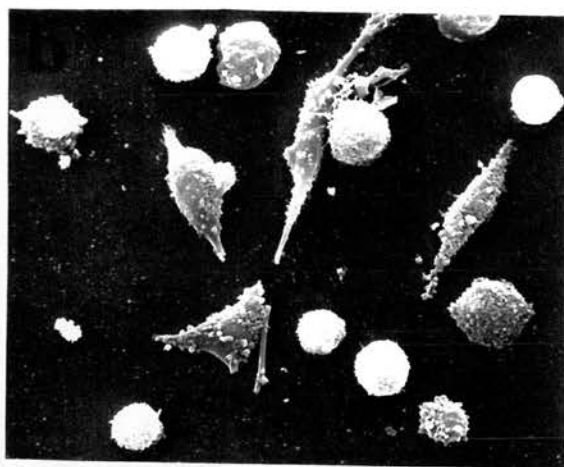
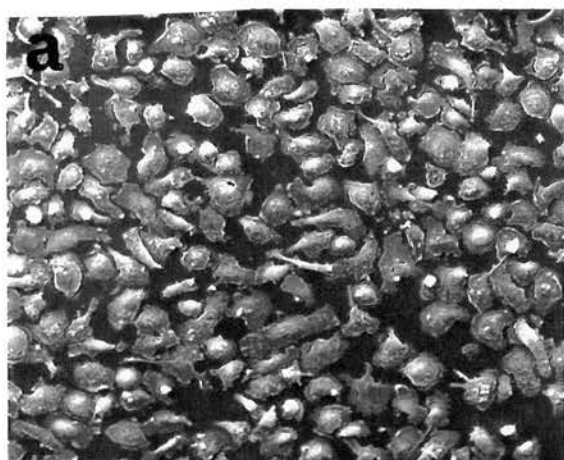


TABLE 3.1 Surface Changes of EV1-Infected MDM Observed under SEM

| postinfection (day) | Cellular condition* (%) | | | cell surface with globular structures (%) |
|------------------------|-------------------------|----------------------|------------------------|---|
| | healthy | mild degeneration | severe degeneration | |
| control | 91.7 | 6.0 | 2.3 | < 3 |
| 2 days | 92.7 | 5.5 | 1.8 | 7.7 |
| 4 days | 80.1 | 14.9 | 5.0 | 36.4 |
| 6 days | 65.1 | 21.5 | 13.4 | 51.1 |
| 8 days | 7.5 | 17.9 | 74.6 | 90.1 |

* Cellular condition was classified by the appearance of surface ruffles, pseudopods and globular structures on MDM surfaces. Values are taken from observations of 200-300 cells.

Figure 3.9 Scanning electron micrographs of MVV-infected AM

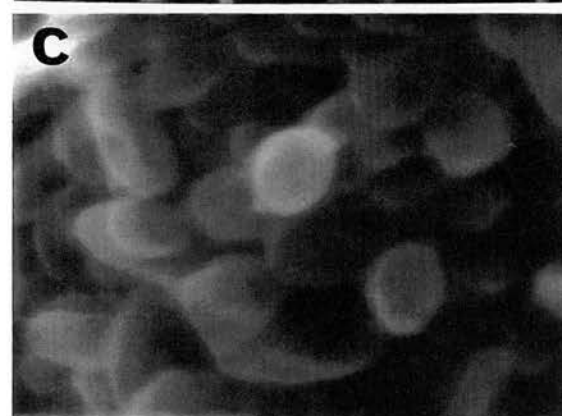
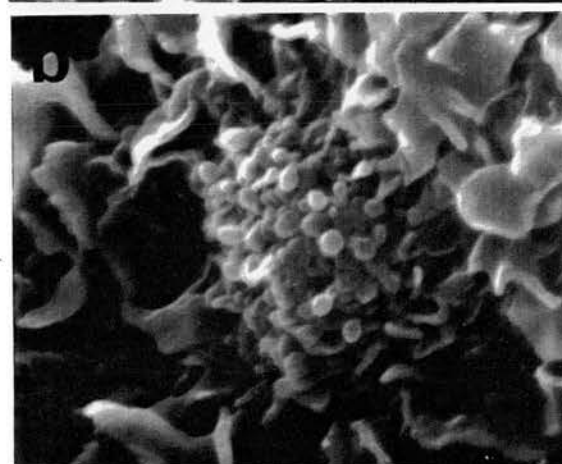
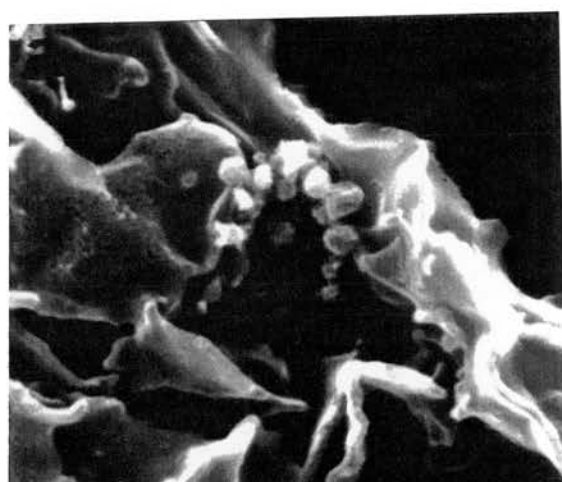
Fresh AM were cultured on coverslips in 24-well plates for 1 day and then infected with MVV, 1 TCID₅₀ per cell. Two days postinfection cells on coverslips were processed as described in section 2.6.

a & b. MVV-infected AM had lost their surface ruffles in specific areas and showed instead a clump of globular structures. In contrast, the surface ruffles surrounding these areas were still intact.

Magnification: x5,000.

c. High magnification of globular structures of (b) which were of 200-250 nm in diameter. Black bar = 1µm.

Magnification: x40,000



1 μ m 30.1 kV 4.00E4 4793/00 384/91

Figure 3.10 Scanning electron micrographs of MVV-infected skin fibroblasts

Skin fibroblasts were cultured on coverslips in 24-well plates and then infected with MVV, 0.5 TCID₅₀ per cell. At designated times, cells on coverslips were processed as described in section 2.6.

a. Uninfected skin fibroblast. Cell surface was flat with little contrast to the coverslip and attached well to the coverslip. The cellular surface was very smooth but with occasional debris.

Magnification: x5,000

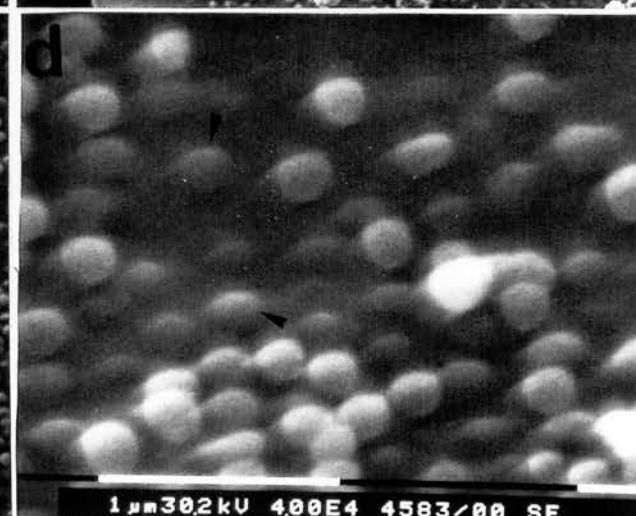
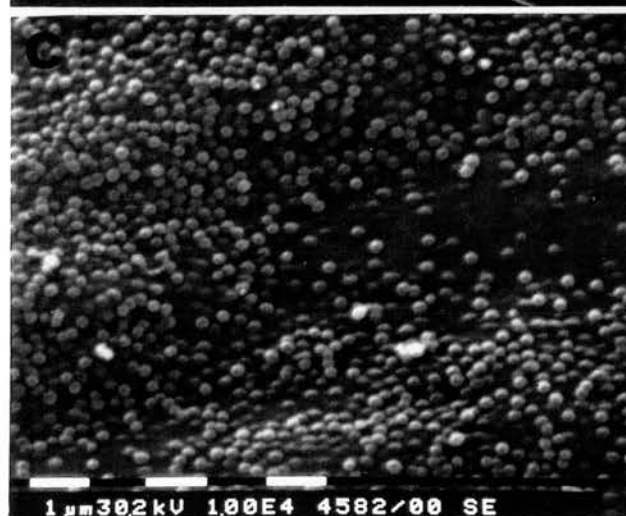
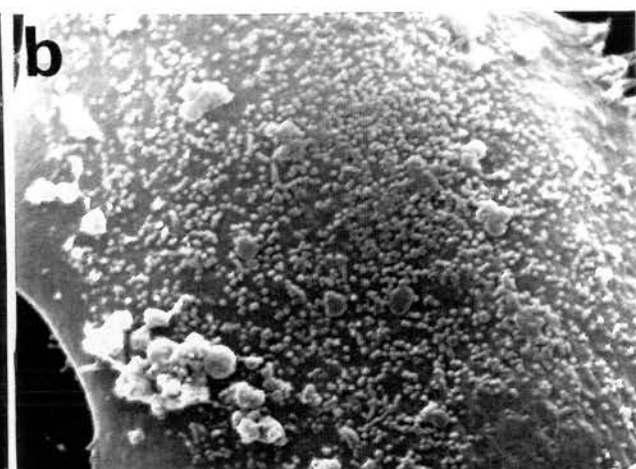
b. MVV-infected skin fibroblast 2 days post infection. The cellular surface was very rough, containing a large number of small globular structures and cell debris of irregular size.

Magnification: x5,000.

c & d. MVV-infected skin fibroblast 4 days post infection. A large number of viral particles with similar size and shape had accumulated on the skin cell surface. Viral particles were 200-250 nm in diameter. It also shows viruses just budding (►) from the cytoplasmic membrane (d). Space bar = 1µm.

Magnification c: x10,000

Magnification d: x40,000



SDS-polyacrylamide gel electrophoresis patterns of the MVV viral proteins were stained using serum from a MVV infected sheep (848A) or control normal sheep serum. The normal sheep serum stained bands at around 60-70KD in both uninfected and infected skin cells (figure 3.11.c). A clear band at about 16 KD was detected with MVV sheep serum in the lysates of uninfected and viral infected AM and as such is unlikely to be virus specific. However, the development of viral specific protein bands could be seen with increasing time post infection (figure 3.11.A) especially the p25 *gag* band (from day 4 post infection) and *env* gp110 smear (from day 6 post infection).

Both lysates of MVV-infected skin cells and AM, and their released virions showed a predominant p25 *gag* band and an *env* smear (from 110KD-135KD) (figure 3.11b).

It was very difficult to compare the *gag* (p25) : *env* protein (gp110) ratio present in the lysates of MVV infected cells and the assembled virions released in the supernatant. However there did not appear to be a specific lack of *env* compared to *gag*.

In addition, other viral specific proteins, 14KD, 17KD, 35KD, 41KD could be detected in the lysates of MVV-infected cells (14KD and 17KD in virion as well). These proteins might include *gag* precursor proteins and processing intermediates and *pol* gene products.

3.3 Discussion

EV1 replication in skin fibroblasts and mature macrophages *in vitro* is rapid and lytic. Therefore EV1 is like the rapid/high MVV isolates reported previously (Lairmore et al., 1987), although EV1 causes less syncytial formation than others. The maturation state of monocytes (time in culture) affected MVV replication with

Figure 3.11 Comparison of viral peptides synthesized in AM and skin fibroblasts

AM (day1) and skin fibroblasts were cultured in T-175 flasks and infected with EV1 at 0.5 and 0.1 TCID₅₀ per cell, respectively. Cells (AM on 2-9 days post infection; skin fibroblasts on 7 days post infection) were harvested, counted and pelleted by centrifugation at 10,000xg for 15 minutes. Virions were further pelleted from clarified supernatant by centrifugation at 100,000xg for 2 hours. Cellular and viral pellets were suspended in a small amount of PBS (4x10⁷ cell/ml). Protein concentration was determined by the Bio-Rad protein microassay.

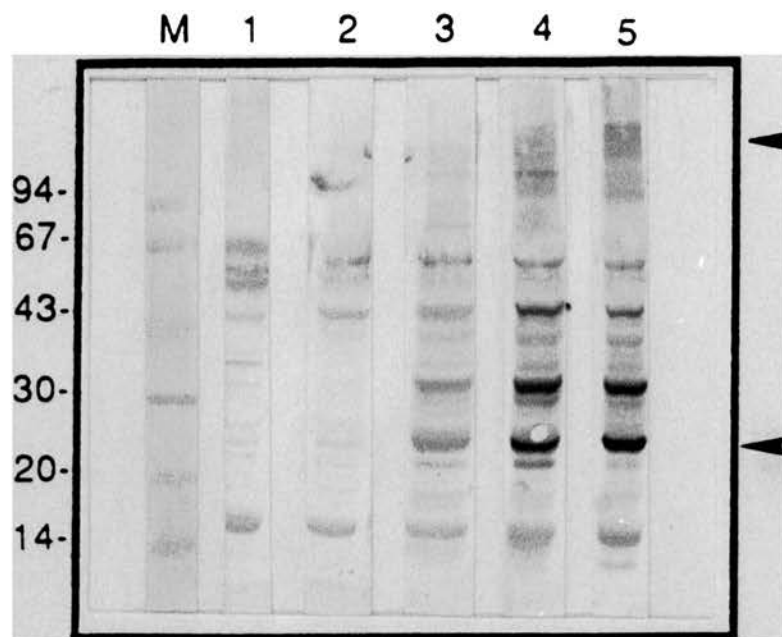
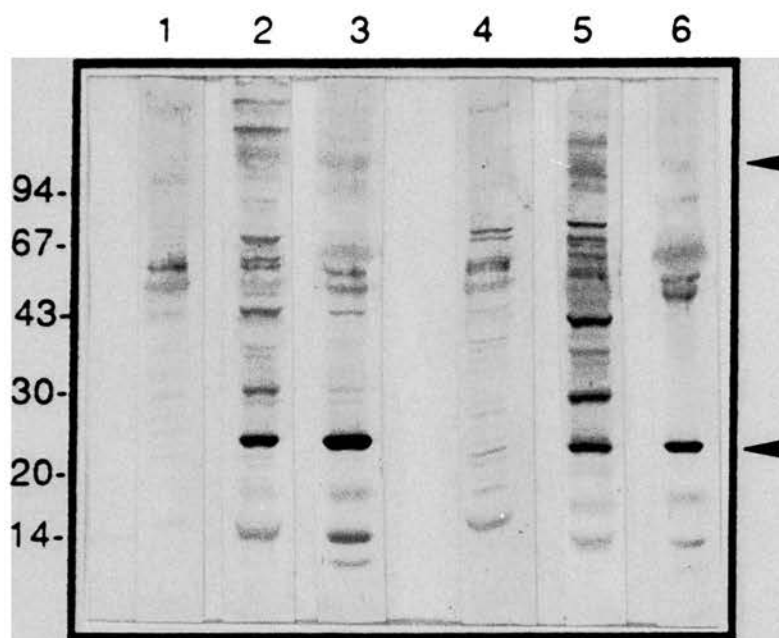
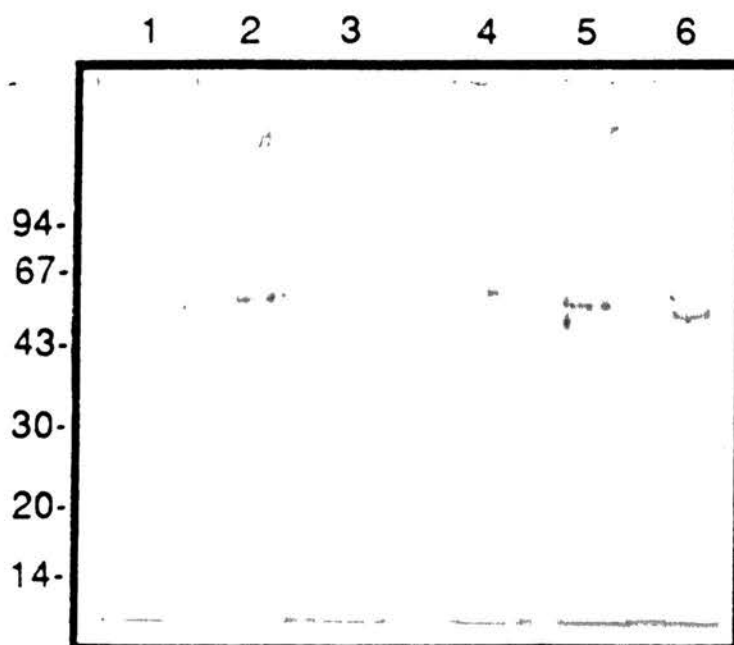
Samples and sample buffer were mixed and boiled for 5 minutes. Five µl of specimen containing 1x10⁵ AM (A) or 5µg protein (B and C) were loaded in each track of 5-20% polyacrylamide gradient gels.

Separated peptides were transferred to nitrocellulose filters and then stained with serum taken from a MVV-infected sheep (848A, panel A & B) or control normal sheep serum (panel C), followed by rabbit anti-sheep IgG-alkaline phosphatase conjugate. The bands were developed in alkaline phosphatase substrate, 5-bromo-4-choloro-3-indoyl-phosphate as described in section 2.4.3.

A. Uninfected AM lysate (lane 1) and EV1-infected AM lysate (lane 2: 2 day pi; lane 3: 4 day pi; lane 4: 6 day pi and lane 5: 8 day pi) were loaded in each track. Molecular weight markers (lane M) and p25 and gp110 positions (▶) are indicated.

B. Comparison of viral proteins expressed in MVV-infected AM lysates and skin fibroblast lysates and found in supernatant virions. Lane 1: Uninfected AM lysate, Lane 2: MVV-infected AM lysate (9 days pi); Lane 3: viral pellet from AM; Lane 4: Uninfected skin fibroblast lysate; Lane 5: MVV-infected skin fibroblast lysate 6 days pi, and Lane 6: viral pellet from skin fibroblasts. Molecular weight markers and p25 and gp110 positions (▶) are indicated.

C. As B but stained with normal sheep serum.

A**B****C**

virus being produced earlier and to high levels in mature macrophages (7 days cultured before infection). This has been previously shown for MVV (Gendelman et al., 1986) and CAEV (Narayan et al., 1983). A similar phenomenon has also been reported for HIV replication in fresh and mature monocytes (Potts et al., 1990). This suggests that fresh monocytes do not support productive lentivirus replication. How the monocyte stops replication is unknown. HIV replication in macrophages apparently causes a more chronic infection than EV1 with more syncytial formation (Perno et al., 1989; Collman et al., 1989; Schmidtmayerova et al., 1992). However, these results are variable depending on the viral isolates and macrophages used.

Ultrastructural observation of MVV grown in tissue culture, in particular sheep choroid plexus cells (SCP), has been widely studied (Thorman, 1961; Coward, 1970; Takemoto, 1971; Macintyre, 1973; Weiland, 1980). Strain EV1 has a similar size, shows bar shaped nucleoids and crescent budding forms at cytoplasmic or vesicular membranes as previously observed in other lentiviruses (Munn et al., 1985; Orenstein, et al., 1988; Weiland et al., 1977; Dahlberg et al., 1981; Gelderblom et al., 1991). However, ICA particles were evident in EV1-infected MDM and AM which have not been mentioned in previous observations of MVV-infected macrophages (Lairmore et al., 1987; Narayan et al., 1982).

ICA particles have been reported in MVV infected sheep testicle cells (Takemoto et al., 1971, Coward et al., 1972), but not found in SCP (Thorman et al., 1961; Macintyre et al., 1973; Weiland et al., 1985) or sheep kidney and liver cells (Thormar et al., 1961). There have also been aberrant ICA-like particles described budding from the endoplasmic reticulum in caprine arthritis encephalitis virus (CAEV)-infected tahr cells (Dahlberg et al., 1981). In addition, ICA particles were found in cultures infected with EIAV (Weiland et al., 1977; Gondø et al., 1978) and SIV (Munn et al., 1985), but not HIV. This suggests that ICA particles may be an assembly form in some lentiviruses. However there is always the danger that

endogenous retroviruses or retroviral genes may be activated by infection with another virus (especially as little is known about endogenous sheep retroviral sequences) (Barban et al., 1984; Herr and Spell, 1985). To address this problem MDM from the same source were infected with 2 different MVV isolates and viral assembly studied by electron microscopy. EV1 showed the accumulation of ICA particles whilst none were seen with 1514. This suggests that MVV infection itself has not activated endogenous retroviral sequences. To prove that the ICA particles were MVV *gag* protein immunogold staining with mouse monoclonal anti-p15 was attempted. Although some gold particles could be seen on ICA particles, staining was weak (data not shown) and so it was not definitively shown that MVV *gag* was in these ICA particles. Therefore it would appear that EV1 has a propensity to form ICA like particles in macrophages rather than skin cells. Why different cell types should cause aberrant virion formation or why two highly conserved *gag* antigens (EV1 and 1514 *gag* 90% homologous; Sargan et al., 1991) should act differently in macrophages is unknown.

ICA particles in retroviruses contain a high molecular weight polyadenylated genomic RNA (Patterson et al., 1978), but little reverse transcriptase (Wong-Staal et al., 1975). These particles also have defects in their envelope gene, do not appear in extracellular spaces and are not pathogenic (Minna et al., 1974). ICA particles budding into the vesicular lumen or being released from the cytoplasmic membrane of macrophages have not been found, which suggests that ICA particles are aberrant forms of virion in MVV. Whether these particles are infectious is unknown.

ICA particles have been documented as precursors of simian immunodeficiency virus (SIV), but these particles clearly bud at the cytoplasmic membrane with both ring or crescent shaped nucleoids and have clearly seen spikes (Munn et al., 1985). Therefore, SIV is morphologically more closely related to type D rather than type C viruses. In contrast, HIV in lymphocytes and monocytes

does not produce ICA particles and buds only with crescent shaped nucleoids (Palmer et al., 1985; Orenstein et al., 1988). This gives HIV a morphological classification with murine type C retroviruses. In contrast, EV1 in MDM and AM produces ICA particles, but they do not bud at the cytoplasmic membrane and they do not appear to mature in vesicular lumens. There are no ring-shaped buds at cytoplasmic or vesicular membranes and the clear spikes on the viral surface like those found on type D retroviruses (Fine et al., 1978) are not seen. From the morphological observations found here, MVV EV1 has features of type B, C and D retroviruses by both viral budding form and ICA particle formation.

In contrast to MVV which has viral budding from the cytoplasmic membrane, HIV virions locate almost exclusively in intracellular vesicles of monocytes or macrophages (Meltzer et al., 1990; Orenstein et al., 1988). These vesicles are morphologically similar to Golgi apparatus-derived and dilated vesicles (Orenstein et al., 1988). Here the origin of dilated vesicles which accumulated viral particles in MVV infected macrophages could not be identified. Clustered viral particles were observed in the extracellular spaces between infected macrophages which suggests that most viral particles which accumulated in vesicles of AM are released into the extracellular space by exocytosis or cell lysis. This was supported by SEM observation (figure 3.9.a & b).

The surface changes seen in MDM or macrophages infected with MVV were linked to the level of cell degeneration and the amount of viral budding from the surface. Two types of globular structures were found on the surface of MVV infected macrophages. Large globular structures, with varying size (>500nm) and shape, may have been degenerative or rounded surface ruffles. Similar changes in surface ruffles have been found by other investigations, including cytomegalovirus-infected (Bruggen et al., 1989), and lipopolysaccharide-treated macrophages (Kang et al., 1992). These changes may be caused by cell depolarization or changes in plasma membrane fluidity and the microfilament cytoskeleton (Jones, 1986). Other

small uniform globular structures measuring 200-250nm which were present on the surface of MVV-infected skin fibroblasts and MDM and are thought to be virions or sites of viral budding similar to those seen in a previous report (Dubois-Dalcq et al., 1976). In this report viral buds are covered by subglobular units which are thought to be viral *env* proteins gradually being added to the viral surface (Sheffield, 1974; Dubois-Dalcq et al., 1976), but these were not seen here. Viewing may be limited by the resolution of the machine.

The size of viral particles measured by SEM is different to TEM. This may be caused by differences in treatment of specimens, the angle of observation under SEM or error in machine measurements. Different sizes from different methods of viewing have been mentioned before (Dubois-Dalcq et al., 1976). Under TEM observation there was only a small proportion of infected MDM showing sites of viral budding at the cytoplasmic membrane (figure 3.8), however, about 50% of MDM (6 days post infection) observed under SEM showed virions on their surface. The difference may arise from the limited observation of cell numbers and cell surface under TEM compared to greater numbers of cells and more cell surface screened under SEM.

MVV infected cells fall into three groups by their patterns of viral budding. These are viral budding from (i) cytoplasmic, (ii) cytoplasmic and vesicular and (iii) vesicular membranes shown here by skin cells, MDM and AM respectively. The pattern of viral budding of MVV in MDM (above) is somewhat different from previous reports of HIV and other MVV isolates which accumulated in vesicles (Narayan et al., 1982; Gendelman et al., 1988; Lairmore et al., 1987; Schmidtmayerova et al., 1992). Research using CD4-bearing cells shows fusion of HIV-infected MDM therefore viral *env* must be expressed at the cell surface (Crowe et al., 1992), which suggests that HIV viral budding could be from the cytoplasmic membrane of macrophages. Therefore, it is possible that budding from the

cytoplasmic membrane by HIV may not be efficiently detected under TEM observation.

The inverted form of viral buds at the cytoplasmic membrane of MDM infected with strain 1514 has not been reported before. Whether a similar phenomenon would also be found in other cultures such as skin fibroblasts and AM infected with 1514 is not clear, but a normal budding form as seen here with EV1 has been reported in 1514 infected sheep choroid plexus cells (Dubois-Dalcq et al., 1976, 1979). In comparison to EV1, strain 1514 has been highly passaged in tissue culture which may have allowed mutation of the virus until these altered assembly forms are seen. Mutant HIV strains which replicate in MDM generating various aberrant viral budding structures and viral particles have also been reported (Klimkait et al., 1990; Peng et al., 1989).

HIV replication and assembly in macrophages is different to that found in T lymphocytes (Munn et al., 1985; Palmer et al., 1985; Orenstein et al., 1988). Immunoprecipitation analysis of HIV proteins shows that in macrophages *env* glycoprotein synthesis is greatly decreased compared to lymphocytes and that virions released from macrophages have a relative lack of *env* compared to *gag* (reviewed in Meltzer et al., 1990). Although MVV has a similar pattern of viral replication and assembly in AM and skin fibroblasts as HIV has in macrophages and T-lymphocytes, the results shown here suggest that *env* protein synthesis is not affected by the host cell type in MVV. The proportion of *env* protein, gp110, assembled in virions or produced in cells was not accurately evaluated, but the clear *env* viral protein band seen in AM lysates or supernatant virus is in direct contrast to immunoprecipitation results from HIV infected macrophages. By electron microscope study mature MVV virions released from both skin fibroblasts and AM showed very short, faint spikes (figure 3.5.d & 3.6.c) on their surface which is probably viral *env* protein.

3.4 Summary

MDM, AM and skin fibroblasts were all permissive for infection by the British isolate of MVV, EV1. Virus grew rapidly and produced high titers of virus in skin fibroblasts and mature MDM. However replication was slower in less mature MDM or AM.

TEM and SEM observation showed that EV1 not only buds at vesicular membranes, but also at the cytoplasmic membrane of MDM. This is different to MVV replication in AM which did not show budding at the cytoplasmic membrane. ICA particles are evident in the cytoplasm of AM and MDM infected with strain EV1, but not MDM infected with strain 1514. The morphological classification of MVV as a type C or B or D of retrovirus, by ICA particle formation, budding form and mature virion structure is therefore complicated by differences in viral strain or infection of different cell types.

There were no gross defects in *env* protein synthesis in MVV-infected AM or *env* incorporation into virions from AM compared to skin fibroblasts.

CHAPTER 4

PART A:

PHENOTYPE OF OVINE MONOCYTE-DERIVED- MACROPHAGES INFECTED WITH MAEDI VISNA VIRUS

PART B:

PHENOTYPIC CHANGES ON ALVEOLAR MACROPHAGES IN SHEEP WITH MAEDI VISNA VIRUS INFECTION

4.1 Introduction

The monocyte/macrophage lineage originates from haemopoietic stem cells which subsequently develop into monoblasts or promonocytes in bone marrow. By complex cytokine stimulation and regulation, these cells differentiate into monocytes and are released into blood. Monocytes may remain in the blood for 36-104 hours and then emigrate into tissues. Subsequently, monocytes differentiate and mature as resident macrophages (Beelen et al., 1989). These resident macrophages have similar morphology and basic phenotype, but still display considerable functional heterogeneity in response to the resident environment (Beelen et al., 1989). Interleukin 3 (IL-3) and granulocyte-macrophage-CSF (GM-CSF) have a broad specific activity on pluripotent stem cells leading to their differentiation and proliferation (Schrader, 1986). For macrophages, IL-3 acts on early progenitors which develop into mature cells of multiple lineages (granulocyte, erythrocyte, monocyte and megakaryocyte). Macrophage-CSF (M-CSF) and GM-CSF act at a late stage to stimulate progenitors to develop into monocytes and macrophages (Geissler et al., 1989; Eischen et al., 1991; Haig et al., 1990).

Over 90% of circulating human monocytes express HLA-DR, whereas, only 25-60% express HLA-DQ and DP (Gonwa et al., 1986). MHC class II on monocytes is closely linked to antigen presentation (Unanue and Cerottini, 1991). The leukocyte integrin family including LFA-1 (CD11a), CR3 (CD11b), P150/95 (CD11c) and fibronectin receptor are major cell surface molecules on monocyte/macrophages (Ross et al., 1989). Intercellular adhesion molecule-1, ICAM-1, serves as the ligand for LFA-1 (Mackay and Imhof, 1993). Both molecules are involved in stabilizing cell contact between macrophages and immune effector cells to allow antigen presentation to lymphocytes (Mackay and Imhof, 1993). Both CD11b and CD11c molecules are specific for complement component, C3b, and are involved in phagocytosis (Myones et al., 1988; Owen et al., 1992).

Immunoglobulin Fc receptors (FcR's) are involved in phagocytosis and endocytosis (Fanger et al., 1989; Sarmay, 1992). Fc γ RI (CD64), Fc γ RII (CDw32) and Fc γ RIII (CD16) have been identified on monocytes and macrophages (Anderson, 1989). Expression of these molecules on macrophages is enhanced by IFN- γ and IL-2 (Fanger et al., 1989; Heagy et al., 1984; Gonwa et al., 1986; Nash et al., 1992). CD14 is an important surface molecule of human monocytes and serves as a receptor for LPS (Ziegler-Heitbrock and Ulevitch, 1993). This molecule on human monocytes is down-regulated by IFN- γ (Ziegler-Heitbrock, et al., 1992).

Monocyte/macrophages play a central role in viral persistence in both HIV and MVV infection, but many functional studies of monocyte/macrophages in HIV infection do not show conclusive results (Gendelman et al., 1989). Not only are results controversial but many functional defects of monocytes during HIV infection could be associated with the defect in T lymphocytes such that the generation of cytokines, in particular, IL-2 and IFN- γ is defective (Murray et al., 1984). There is often variation in monocyte/macrophage phenotype amongst individuals as well as in macrophages derived from different tissues. These differences may be related to differences in function. Therefore the study of changes in monocyte phenotype during culture may help to explain functional changes.

It has been reported that there is a decrease in HLA-DR expression in monocytes from HIV-infected patients with AIDS (Heagy et al., 1984; Roy et al., 1987). In addition, AM from ARC and AIDS patients did not express the normal amount of class II and LFA-1 antigen on their surface when compared to the normal controls (Roy et al., 1987; Bray et al., 1993). Documentation shows that there is a great reduction in MHC class II expression and function of dendritic cells and Langerhan's cells in HIV-infected patients with ARC and AIDS (Eales et al., 1988; Belsito et al., 1984). *In vitro*, studies using the human promonocytic cell line U937 as a monocyte model have shown an increase in the expression of mature monocyte surface markers, except for HLA class II antigens, after infection with HIV (Petit et

al., 1987). It is thought that this has contributed to the immunosuppression of HIV infection.

Although there are many biological similarities between HIV and MVV infections, particularly in viral persistence (Narayan and Clements, 1989), the fate of the surface molecules expressed on macrophages, *in vivo*, is different. It has been shown that MHC class II expressed on the surface of AM from MVV-infected sheep is significantly increased compared to uninfected sheep (Lujan et al., 1993; Cordier et al., 1990). Similarly, an increase has been reported in macrophages in synovial fluid from MVV-infected sheep (Kennedy-Stoskopf et al., 1989b; Harkiss et al., 1991). Furthermore, a significant increase in spontaneous fibronectin released by AM from maedi has also been described (Cordier et al., 1990). This suggests that macrophages from MVV affected sites are activated rather than being decreased functionally as in HIV. These differences are thought to be an indirect effect caused by LV-IFN which is generated during MVV infections (Narayan et al., 1985; Zink et al., 1989). However, the direct effect on surface molecules of macrophages after infection with MVV *in vitro* is not clear. Therefore, a study of the changes in surface molecule expression on cultured MDM infected with MVV and the comparison with changes in AM from MVV-infected sheep was undertaken. Surface molecule expression on cultured monocytes during maturation will also be discussed as well as the general properties of cultured monocytes (monocyte-derived macrophages, MDM).

4.2 Results

Part A: Phenotypic Study of Monocyte-derived Macrophages

4.2.1 General observation of cultured monocytes during maturation *in vitro*

Gelatin-coated flasks or plates were used to culture monocytes from PBMC as previously described in section 2.2.3. The numbers of monocytes recovered by different culture methods were compared. Monocytes recovered from the flasks in which non-adherent cells had been washed off after 1 or 2 hours incubation showed high purity (70-80%) based on non-specific esterase staining, but low absolute numbers after 3 to 5 days in the culture. In contrast, higher numbers but lower purity monocytes were recovered in adherent cells from flasks in which PBMC had been left overnight and then the non-adherent cells were removed. Many of the contaminating PBMC were trapped on the flasks by fibrinous strands. After 5 to 7 days of culture, these fibrinous strands with the contaminating cells and other debris could be easily removed from the flasks. The purity of monocytes recovered from these cultures was near or over 95%, confirmed by Giemsa and nonspecific esterase staining (figure 4.1). Monocytes cultured for 5-7 days markedly increased their size and cytoplasm/nucleus ratio and also showed very strong nonspecific esterase (NSE) activity compared to fresh monocytes. Mild to moderate proliferation of MDM could be found in cultures, but it was very variable from culture to culture. If present, this proliferation persist for 2-3 weeks and their cellular density in long term cultures gradually decreased. Most macrophages from proliferating cultures which had fallen off the flask could be passaged to another flask where they became adherent. Using these methods, MDM could be continuously cultured for at least 3 months. However, fibroblast, epitheloid and syncytial cell contamination also appeared in long term cultures which could interfere with experiments.

Figure 4.1 Monocyte differentiation and maturation in culture

Monocytes were cultured in gelatin-coated flasks or 24-well plates in RPMI-1640/10% FCS/10% LS as described in materials and methods (section 2.2.3).

A. Seven day cultured MDM, which looked confluent, were fixed in methanol and stained with Giemsa's stain. The differentiated macrophages had a round or spindle shape and a high cytoplasm/nucleus ratio. The spindle shaped macrophages would become round in prolonged culture.

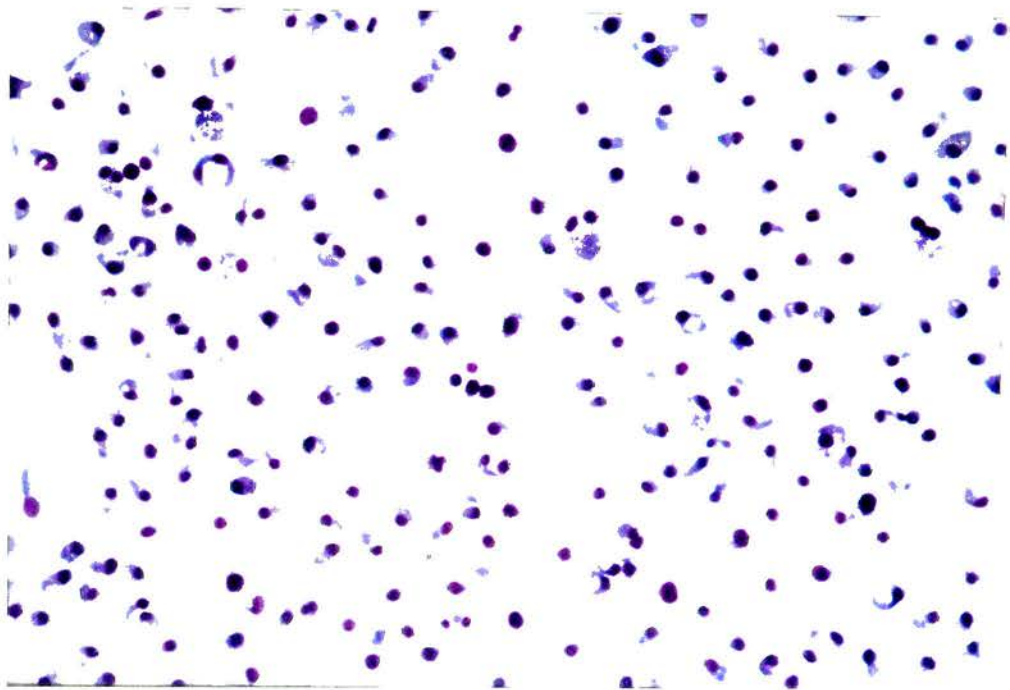
Magnification: x200

B. Seven day cultured MDM were non-specific esterase (NSE) positive using α -naphthyl acetate (section 2.2.5). The NSE staining intensity was dependent on the maturity of the macrophages. The maturity of the macrophages was also indicated by the cytoplasm : nucleus ratio.

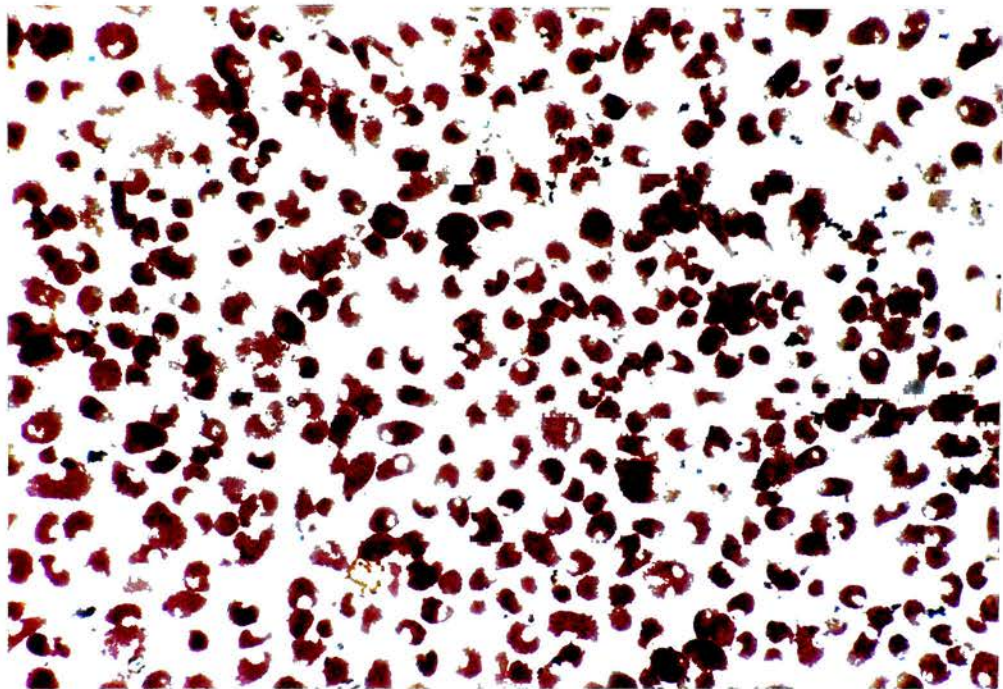
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FIGURE 4.1

A



B



4.2.2 Kinetics of surface molecule expression on cultured monocytes of sheep

Since the cultured MDM were variable in size, MDM were gated on a large FSC and SSC area excluding lymphocytes and cell debris. Analysis of cell staining from the FACS often uses a 1% gate set on negatively stained cells to calculate the percentage of cells above this as positively stained cells with the antibody of interest. Using the percentage positive cells to express the phenotype of MDM may not reflect the actual situation, as with monocyte maturation there is an increase not only in size, but also autofluorescence of the cells (Pankow et al., 1990). Due to these factors, it was very difficult to separate positive and negative populations of MDM, particularly where molecules were weakly expressed. Hence, the mean fluorescence intensity (MF) of specific molecules with negatively stained fluorescence (normal mouse serum, NMS) subtracted was used to express the following data.

VPM32 is a monoclonal antibody which detects tissue macrophages, but not monocytes or T and B lymphocytes (Gonzalez, 1988). The antibody is thought to be specific for Fc γ RIII of macrophages (Gonzalez, 1988). With monocyte maturation *in vitro*, the expression of VPM32 antigen were greatly increased from day 3 (MF=34.8) until day 9 (MF=43.2) and decreased slightly on day 13 (MF=28.4) (Figure 4.2). Setting a 1% gate on NMS staining, the average number of positive cells on day 9 were between 60% and 80% of cultured macrophages.

VPM19 is a monoclonal antibody specific for a monomorphic region of MHC class I molecules which is expressed on most nucleated cells (Hopkins and Dutia, 1990). Monocytes express class I throughout differentiation *in vitro* to macrophages (figure 4.2) although the fluorescence intensity of staining and therefore possible density of MHC class I on the surface appeared to decrease within the first 3 days of culture to steady levels thereafter. VPM36 and VPM38 are monoclonal antibodies specific for MHC class II DQ and DR α chains, respectively (Dutia et al., 1990). The expression of MHC class II antigen was moderately high

(MF=85.6; 60-80%) on fresh monocytes, but after 3 to 5 days in culture, the MHC class II molecules were dramatically reduced such that less than 5% cells (MF=6.4) were positively stained. Monocytes from sheep which had been primed with antigen in complete Freund's adjuvant or monocytes cultured without removing the lymphocytes (nonadherent cells) maintained a low to moderate density of MHC class II expressing positive cells (10-30%) (data not shown). MHC class II DQ was expressed in a similar manner and intensity as MHC class II DR on fresh and cultured monocytes.

Fresh monocytes did not express either CD4 or CD8. After culture, they gradually began to express both molecules on their surface (figure 4.2) with maximal levels around day 7 in culture (MF: CD4=25.1; CD8=35.6) which began to decrease after day 9. Apparently, more CD8 was expressed than CD4.

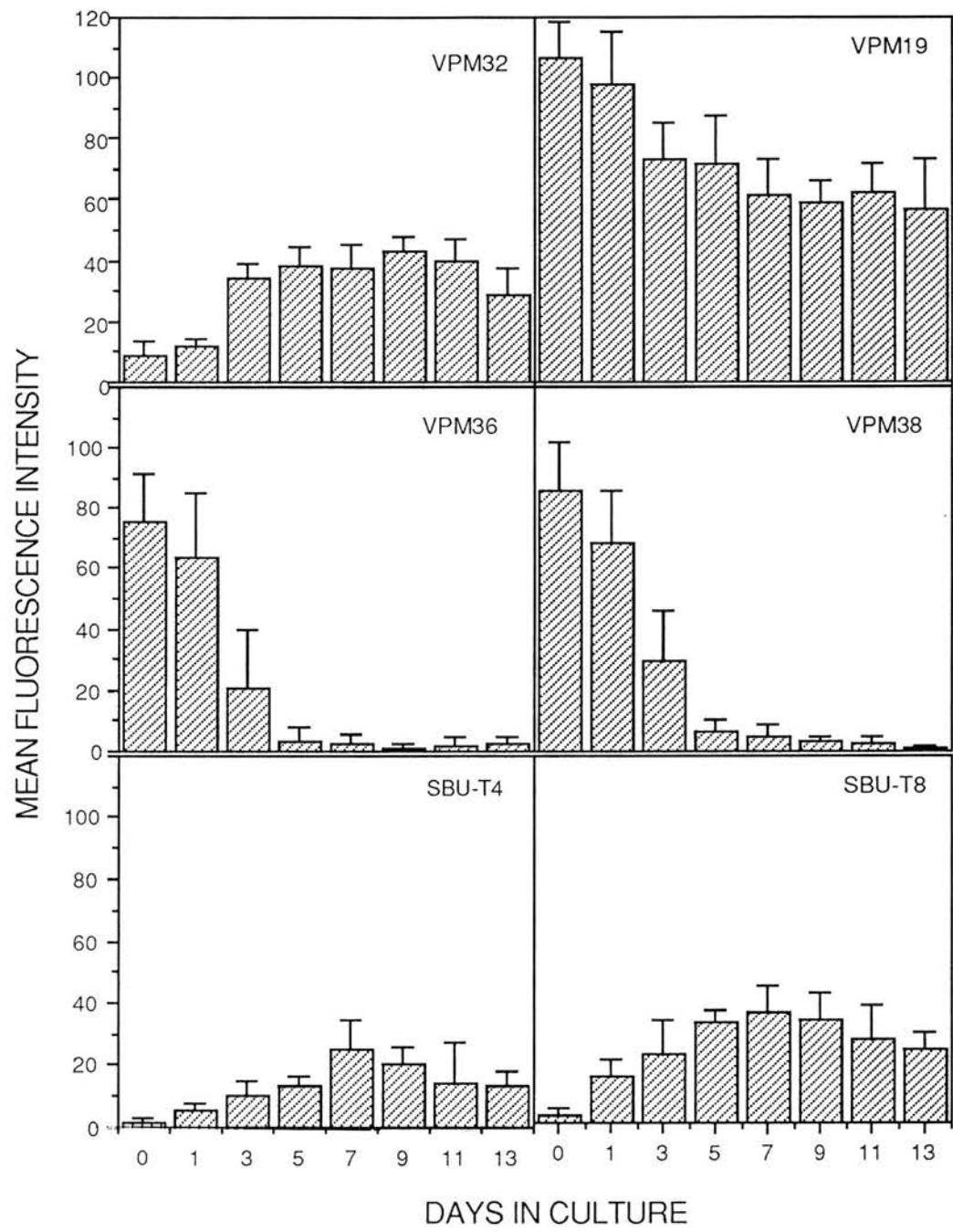
F10-150-39 (anti-LFA-1) and L180-1 (anti-LFA-3) monoclonal antibodies stain lymphocytes, monocytes and macrophages. It was, therefore, difficult to differentiate lymphocytes and monocytes in fresh PBMC using these monoclonal antibodies. However, with the increased size and purity of cultured monocytes, these monoclonal antibodies could be used to analyze the surface molecules on MDM. The percentage of mature MDM positive for LFA-1 and LFA-3 on day 7-12 of culture was maintained between 50-60% (MF=46.2-54.4) and 70-80% (MF=50.3-63.5), respectively (data not show). VPM 8 is a monoclonal antibody specific for Ig L-chain (Dr. P. Bird; personal communication). The number of VPM8 positive cells decreased during monocyte maturation, but at the end of the culture on day 13, 15% (low intensity) of cultured MDM were still positive for VPM8 antigen. However, the intensity varied greatly between cultures (data not shown). Another monoclonal antibody, VPM65, has been newly developed in the Department and reacts to the CD14 molecule, the LPS receptor, on monocyte/macrophages (Gupta et al., 1993). Both fresh monocytes and cultured

Figure 4.2 Phenotype of cultured monocytes

Monocytes from uninfected sheep were cultured in gelatin-coated flasks as previously described (section 2.2.3). Non-adherent cells were removed after 2 hours (for day 0 assay) or overnight. Adherent cells (monocytes) were harvested on designated days by treating cells with 5mM EDTA in PBS. Cells were counted and stained with monoclonal antibody (as indicated) and then rabbit anti-mouse IgG, F(ab)₂ fragment-FITC conjugate. Cells were analyzed immediately by flow cytometry.

The results have been expressed as mean fluorescence intensity (MF) which is (MF with monoclonal antibody - MF with NMS). The data was collected from 5 separate experiments.

FIGURE 4.2



MDM showed a moderately high intensity (MF=51.4 on day 7) of staining for the LPS receptor without much change during culture (data not shown).

4.2.3 Regulation of the MHC class II phenotype of cultured macrophages

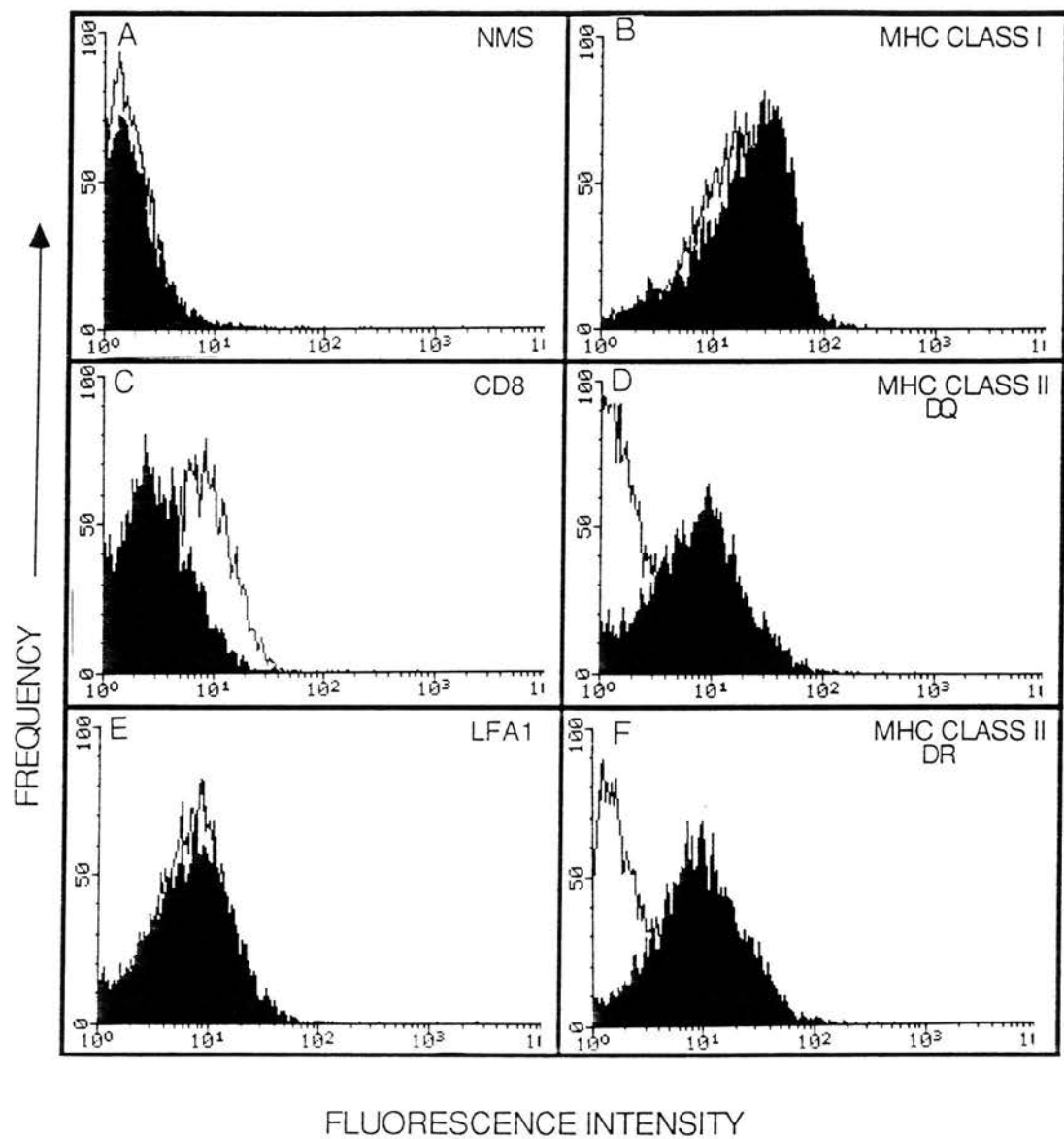
We were interested in using MDM in antigen presentation assays and so the loss of MHC class II expression during culture was unfortunate. It was therefore decided to see if MHC class II expression could be induced easily on cultured MDM. A crude Con A-supernatant generated from cultured lymphocytes from the mesenteric lymph node (section 2.7.3) was used to activate cultured MDM. In this experiment MDM were cultured as previously described and then different concentrations of Con A-supernatant (10%, 25%, 50%, and 75% v/v) were added to the MDM culture for 1 to 3 days and cells harvested for phenotypic analysis. A representative profile is shown in figure 4.3. Results show that the crude Con A-supernatant (50% v/v) greatly enhanced MHC class II expression (MF: DQ=38.41; DR=44.63) after 2 days treatment, compared to untreated MDM (MF: DQ=5.5; DR=13.8). Treatment of the cells with Con A-supernatant for 1 day only slightly enhanced MHC class II density on MDM. Treatment for 3 days did not greatly improve class II expression over 2 days treatment (data not shown). In addition, MDM treated with phytohemagglutinin (PHA; 10 μ g/ml) or Con A (5 μ g/ml) alone for 2 days did not enhance MHC class II expression (data not shown). Con A-supernatant slightly enhanced MHC class I expression on MDM, but the intensity of CD8 molecules expressed on cultured MDM was slightly decreased. Other molecules including LFA-1 and LFA-3 were not affected (Figure 4.3).

Figure 4.3 Regulation of surface molecule expression on cultured MDM by Con A-supernatant

Con A-supernatant was prepared from the culture supernatant of mesenteric lymph node stimulated with 5 μ g Con A/ml for 2 days as described in section 2.7.3. Twelve-day cultured MDM were fed with 50% Con A-supernatant (v/v) for 2 days. Cells were harvested and stained with monoclonal antibodies as previously described (section 2.7.2). A: NMS; B: VPM19 (anti-MHC class I); C: SBU-T8 (anti-CD8); D: VPM36 (anti-MHC class II DQ); E: F10-150-39 (anti-LFA-1); F: VPM38 (anti-MHC class II DR).

The unshaded area (□) shows cultured MDM and the shaded area (■) shows MDM treated with 50% Con A-supernatant.

FIGURE 4.3



4.2.4 Affect of MVV infection on MDM phenotype

Two methods were used to look at the effect of MVV infection on MDM phenotype. In the first experiment, MDM were infected with MVV, 1 TCID₅₀ per cell, on day 3 of culture, then virus-infected and mock-infected MDM were harvested for phenotypic analysis every 2 days (data not shown). There was often variation in the expression of CD4, CD8, MHC class I and VPM32 antigen at 2 days postinfection, but this included both increased and decreased levels of expression. After 4 days postinfection, the fluorescence intensity of most surface molecules on MVV-infected MDM was similar to those found on mock-infected MDM. More than 4 days post infection, most surface molecules, especially MHC class I, CD8 and anti-VPM32 antigen were greatly decreased, this could be by up to 30-40% on 7-8 days MVV-infected MDM compared to the uninfected control. However, owing to the great variation in surface molecule staining in different cultures, these results did not show statistically significant differences between virus-infected and mock infected MDM (data not show). Both MVV and mock infected MDM were negative for MHC class II expression unless given prior treatment with Con A-supernatant.

Previous observations of MDM-infected with MVV (chapter 3) have shown that after 6 days there is a lytic infection of MDM such that most cultured MDM are gradually lost. In the above experiment, it was difficult to get enough cells for flow cytometric analysis in late MVV-infected cultures. Moreover, the decreased expression of surface molecules 7-8 days postinfection was probably due to cell death caused by productive replication of virus within cells. Therefore, a second experiment was designed to address the early phenotypic changes on MDM infected with MVV. In this experiment monocytes were cultured then infected with MVV on day 7, 9, 10, and 11 individually. Some cultures were also infected with MVV as early as 5 days. Antigen expression on MVV and mock infected MDM was then analyzed on the same day, day 12. The results are shown in table 4.1 and figure 4.4.

Expression of most surface molecules including CD4, CD8, VPM32, MHC class I and MHC class II on infected MDM was not significantly altered up to 5 days post infection (table 4.1). However, some experiments showed a tendency for surface molecule expression to increase slightly at early times after infection (up to 3 days post infection). After 7 days postinfection, some surface molecules, especially VPM19 and VPM33 which were highly expressed on cultured MDM, were greatly decreased at the cell membrane about 30-40% of values of mock-infected MDM (data not shown).

4.2.5 Detection of viral antigens expressed on the cell surface and in the cytoplasm by flow cytometry

In order to study the relationship between phenotype alterations and viral antigen expression on MDM, viral antigen (*gag* p15) expressed on the cell surface or within the cytoplasm accumulated intracytoplasmically was assayed using flow cytometry. Viral antigen (p15) expressed on the cell surface was detected on unfixed cells as previously described (section 2.7.2). Detection of internal viral antigen was performed by fixing cells in 80% methanol. MVV-infected MDM did not express *gag* antigen on the cell surface up to 4 days postinfection (figure 4.5 E). However, at 6 days postinfection, a small population (14%) of MDM express *gag* antigen on their surface (figure 4.5.F). In contrast, internal viral antigen (p15) could be detected as early as 3 days postinfection (6.7%, data not shown). By 6 days postinfection, 65% of MDM were positive for p15. Interestingly, MVV-infected AM did not express *gag* antigen (p15) on their surface after 7 days postinfection, but more than 60% AM showed internal antigen (p15) (data not shown). In contrast, skin cells express viral antigen (p15) weakly on their surface by 4 days post infection (figure 4.5 B), but cytoplasmic *gag* antigen was strongly expressed as early as 2 days postinfection (figure 4.5 C).

Figure 4.4 Affect of MVV infection on MDM phenotype

MDM were cultured in gelatin-coated T-25 flasks and then infected with MVV, 1 TCID₅₀ per cell on day 7, 9, 10, and 11, respectively. On day 12 MDM were harvested and stained with a panel of monoclonal antibodies: SBU-T8 (anti-CD8), VPM19 (anti-MHC class I); VPM38 (anti-MHC class II DR), VPM32, VPM33, and F10-150-39 (anti-LFA-1) as indicated and subsequently with rabbit anti-mouse IgG, F(ab)₂ fragment-FITC conjugate as described in materials and methods (section 2.7.2).

This is a representative profile of 5 separate experiments. Negative control staining was uninfected MDM stained with NMS (dense dotted line; ······) and has been shown with MVV-infected (solid line; ———) and uninfected (loose dotted line; ·····) MDM stained with specific monoclonal antibodies.

FIGURE 4.4

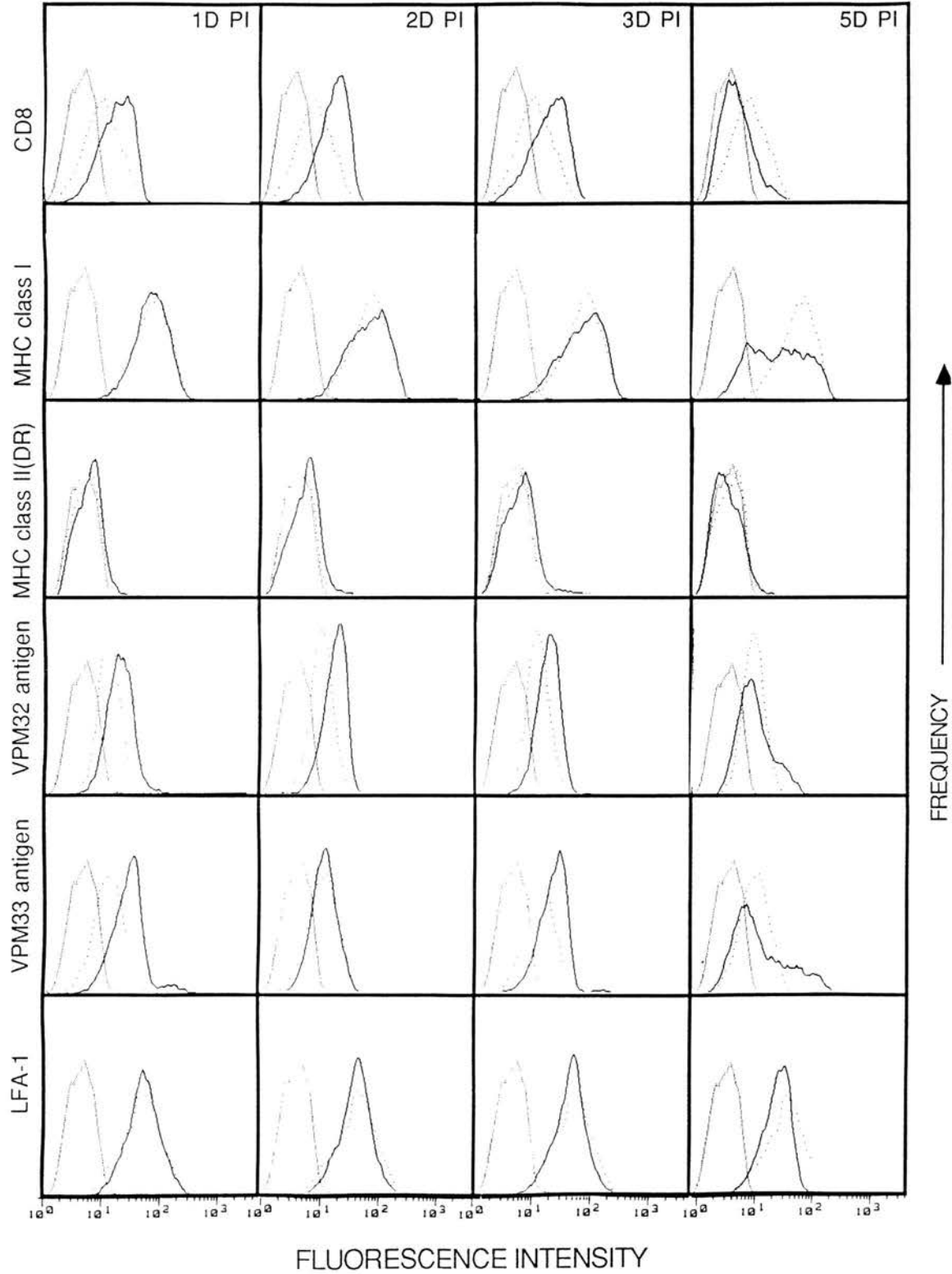


TABLE 4.1 The Comparison of Mean Fluorescence Intensities between MVV and Mock Infected Monocyte-Derived-Macrophages**

| surface molecules | Time postinfection | | | | |
|-------------------|--------------------|-------------|-------------|-------------|-------------|
| | control | 1 day | 2 days | 3 days | 5 days |
| CD4 | 10.9 ± 3.1* | 12.0 ± 1.2 | 17.4 ± 4.9 | 14.2 ± 5.5 | 7.3 ± 6.0 |
| CD8 | 29.7 ± 6.9 | 32.4 ± 6.1 | 38.7 ± 7.9 | 40.0 ± 8.0 | 23.4 ± 4.3 |
| MHC classI | 70.8 ± 7.3 | 73.0 ± 8.2 | 80.9 ± 9.8 | 74.0 ± 11.7 | 63.7 ± 8.5 |
| MHC classII (DR) | 10.4 ± 14.4 | 13.1 ± 6.0 | 5.2 ± 7.0 | 5.9 ± 4.3 | 1.2 ± 10.2 |
| LFA-1 | 47.4 ± 10.3 | 52.0 ± 6.9 | 50.9 ± 6.72 | 51.9 ± 6.9 | 46.2 ± 13.9 |
| LFA-3 | 51.4 ± 6.0 | 54.5 ± 8.6 | 55.5 ± 5.14 | 51.3 ± 7.1 | 45.8 ± 14.9 |
| VPM32 antigen | 39.4 ± 5.0 | 41.9 ± 6.9 | 44.5 ± 13.9 | 46.4 ± 8.4 | 37.6 ± 7.9 |
| VPM33 antigen | 72.5 ± 2.6 | 77.2 ± 15.9 | 78.5 ± 18.1 | 68.2 ± 9.2 | 52.2 ± 9.6 |

* Data (mean ± sd) are expressed as mean fluorescence intensity (MF) where negative control fluorescence (NMS control) has been subtracted. Data were collected from 5 separate experiments.

** Cultured MDM on day 7, 9, 10, and 11 were infected with MVV, 1 TCID₅₀ per cell, and analyzed on day 12.

Figure 4.5 Expression of viral antigen (*gag* p15) on the cell surface or in the cytoplasm of MDM and skin fibroblasts

Cell surface (external) *gag* antigen was detected on skin fibroblasts and MDM blocked with 2% normal rabbit serum and normal sheep serum in PBSA and then stained with monoclonal antibody, 415, followed by rabbit anti-mouse IgG, F(ab')₂ conjugate as described in section 2.4.2. In contrast, cells initially fixed in 80% methanol, blocked with 2% normal rabbit serum and 2% normal sheep serum in PBSA and then stained with monoclonal antibody, 415, followed by FITC conjugate, were used to detect viral antigen in the cytoplasm (internal). Both groups of stained cells were analyzed by flow cytometry.

The upper panel shows skin fibroblasts (A-D) infected with MVV, 0.5 TCID₅₀ per cell and the lower panel shows MDM (E-F) cultured for 7 days, infected with MVV, 1 TCID₅₀ per cell. Infection times are indicated on the figure.

Negative staining with an irrelevant mouse IgG_{2a} monoclonal antibody is shown by the unshaded area (□) with staining for *gag* p15 shown by the shaded area (■). A 1% gate was set on the negatively stained cells and the percentage cells staining positive for *gag* above this is shown in each panel.

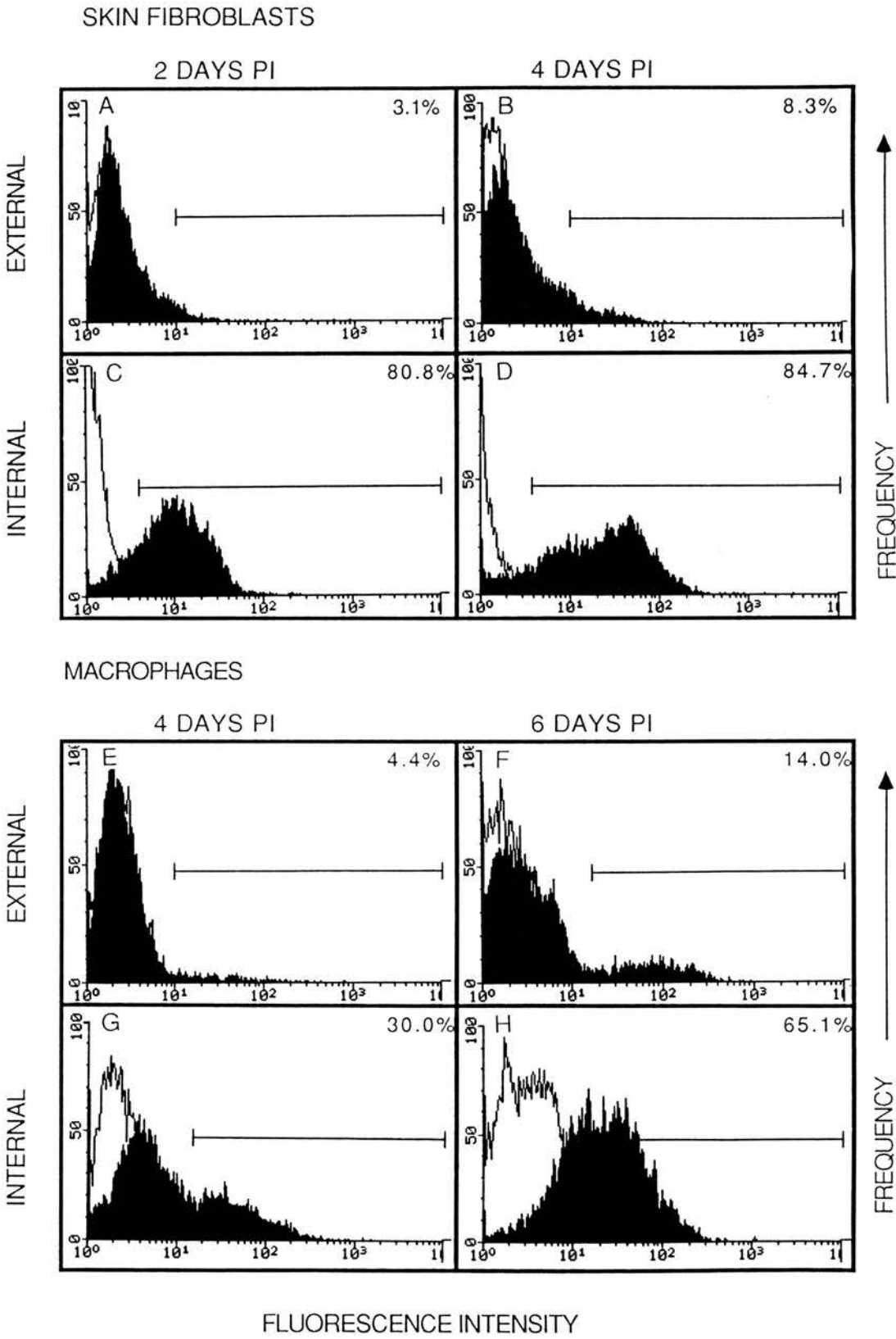
A & B: skin fibroblasts with external *gag* antigen.

C & D: skin fibroblasts with internal *gag* antigen.

E & F: MDM with external *gag* antigen.

G & H: MDM with internal *gag* antigen.

FIGURE 4.5



Part B.

4.2.6 Comparison of the alveolar macrophage (AM) phenotype from MVV-infected and uninfected sheep

In this study lungs from the majority of experimentally infected sheep (13/16) did not show gross pulmonary lesions, whilst those from naturally infected sheep (5/5) showed mild to marked interstitial pneumonia. The lungs with lesions were larger, firmer and heavier than normal lungs. They had a reddish-pink hue with multiple gray foci or were a diffuse gray in color. Control sheep were experimentally culled sheep without infection and showed no gross pulmonary lesions. MVV infected and control sheep were not age and breed matched.

AM from MVV infected sheep, either with or without pulmonary lesions, showed significant increases in the surface expression of MHC class II, DQ and DR, compared to uninfected control sheep (table 4.2 and figure 4.6). AM from lungs showing lesions also had a significantly increased MHC class I expression. However this difference was not seen on AM from lungs without gross lesions (table 4.2, figure 4.6).

AM also expressed LFA-1 and LFA-3. LFA-1 expression significantly increased on AM from lungs with lesions but not on those from lungs without lesions (table 4.2, figure 4.6). LFA-3 expression was unaltered by MVV infection (data not shown). VPM32 staining on AM was very low and showed no change in MVV infected sheep. AM were negative for CD4, CD8 and T19 molecules (data not shown).

4.2.7 Changes in the CD4/CD8 lymphocyte ratio in bronchoalveolar lavages from MVV-infected sheep

Using FSC and SSC parameters to gate on small lymphocytes, the CD4 and CD8 T lymphocyte population was also studied in BAL's. By using the percentage positive lymphocytes to calculate the CD4/CD8 ratio it was shown that there was a

statistically significant decrease in the CD4 : CD8 ratio in MVV-infected sheep with lung lesions compared to uninfected sheep (table 4.2). Most MVV-infected sheep with pulmonary lesions showed a decrease in the CD4 : CD8 ratio (< 1), but the ratio in MVV-infected sheep without pulmonary lesions was variable (ranged from 0.4 - 5.4). In contrast, the CD4 : CD8 ratio of lymphocytes from normal lungs were consistently higher than 3.

4.3 DISCUSSION

4.3.1 Phenotypic expression of cultured MDM

In vivo the migration of monocytes into tissues results in the generation of organ-specific populations of resident macrophages which show different phenotypes and functions (Andreesen et al., 1990). For example AM express high MHC class II levels, but low levels of CD14, whilst peritoneal macrophages show low MHC class II levels but have high CD14 expression (Andreesen et al., 1990). The factors attributing to these differences *in vivo* may be intrinsic differentiation pathways of monocytes influenced by organ specific microenvironments. *In vitro*, it is possible to alter culture conditions (by the addition or absence of serum, growth factors or cytokines) to grow monocytes in different differentiation states and showing different levels of surface antigen expression and function (Geissler et al., 1989; Eischen et al., 1991; Kreutz et al., 1992). The *in vitro* states are therefore comparable to different organ specific macrophages seen *in vivo*.

Serum contains many factors important for monocyte differentiation *in vitro*. In fact monocytes cultured in the absence of serum cannot mature and die in culture. They can, however, be rescued by the addition of growth factors (Geissler et al.,

Figure 4.6 Phenotype of AM from MVV-infected sheep

1×10^5 AM were stained with monoclonal antibodies followed by anti-mouse IgG, F(ab)₂ conjugate as previously described in section 2.7.2.

This is a representative profile of surface molecule expression of AM from MVV-infected sheep with pulmonary lesions compared to uninfected controls. The unshaded area (□) indicates AM stained with normal mouse serum, whilst, the shaded area (■) shows AM stained with specific monoclonal antibodies. VPM36, anti-MHC class II DQ (A & E); VPM38, anti-MHC class II DR (B & F); VPM19, anti-MHC class I (C & D) and F10-150-39, anti-LFA-1 (D & H).

FIGURE 4.6

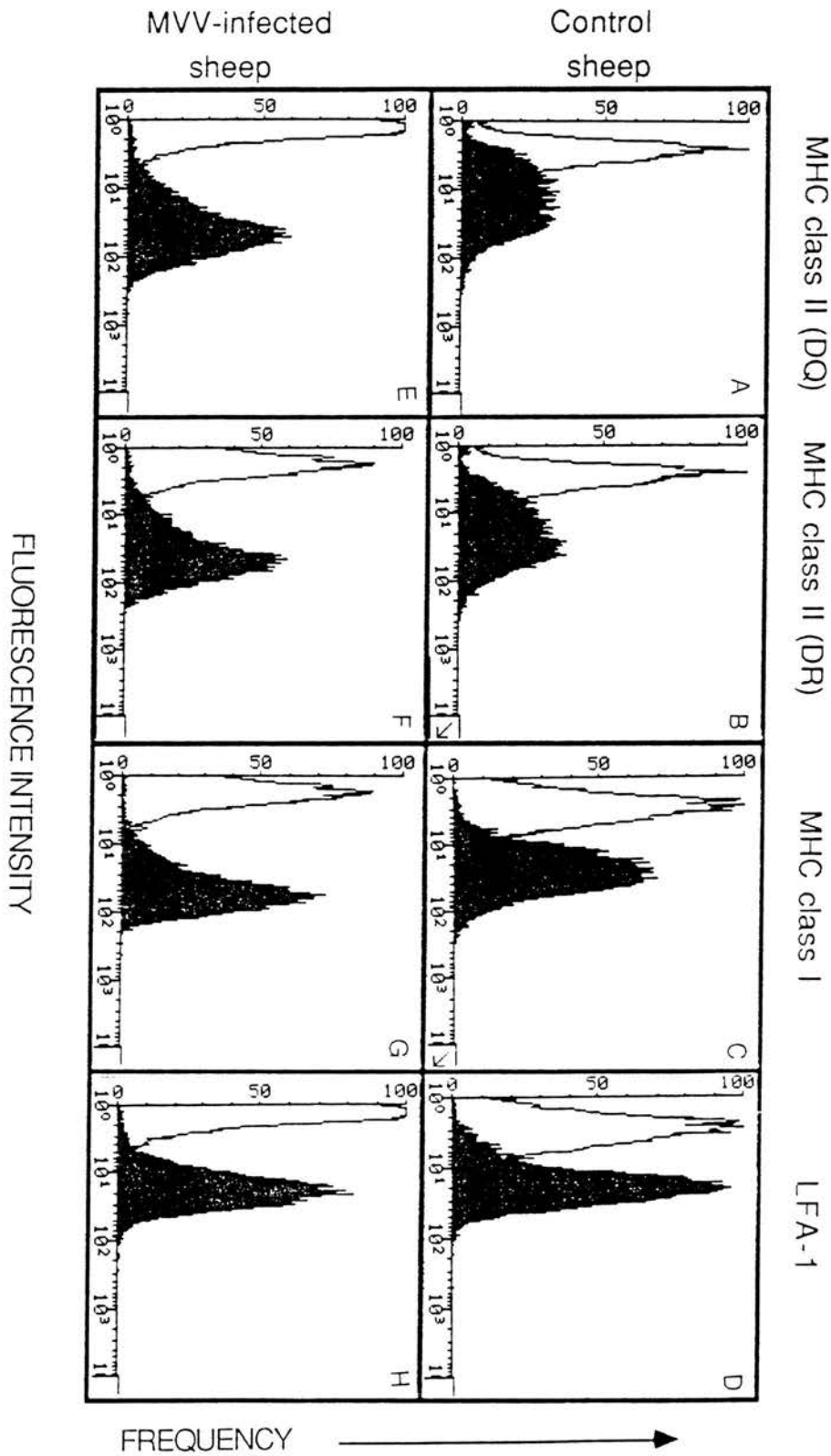


TABLE 4.2 Surface Molecule Expression on BAL Fluid Cells from MVV-Infected and Control Sheep

| Groups | Alveolar Macrophages | | | Lymphocytes | |
|---|--|--|---|---------------------------------------|-----------------------------------|
| | MHC class I (DQ) | MHC class II (DR) | MHC class II (DR) | LFA-1 | CD4/CD8# |
| Control sheep (N=10) | 63.1±12.6* (42.1-79.2)** | 45.7±13.2 (19.5-61.5) | 56.4±9.0 (45.7-69.5) | 51.2±12.3 (34.7-67.8) | 3.2±2.1 (1.9-6.5) |
| Infected sheep without lung lesions (N=13) | 78.6±15.1 (50.5 - 101.5) | 55.2±13.5 ^a (33.6-78.5) | 67.5±12.7 ^a (45.6-89.0) | 57.8±9.4 (43.2-68.1) | 2.5±1.5 (0.4-5.4) |
| Infected sheep with lung lesions (N=8) | 89.4±13.3 ^a (70.6-104.1) | 84.5±8.7 ^{a,b} (71.6-92.7) | 84.9±11.9 ^{a,b} (63.9-97.9) | 64.9±10.5 ^a (47.1-76.0) | 0.7±0.3 ^a (0.4-1.4) |

* Values (mean ± SD) have been expressed as mean fluorescence intensity (MF) (MF with Mab - MF with NMS).

** Range of mean fluorescence intensity

Ratio of alveolar lymphocytes in bronchoalveolar lavage fluid.

^a indicates a statistically significant difference (p<0.05) between MVV-infected and uninfected sheep analyzed by Mann Whitney two tail test.

^b indicates a statistically significant difference between MVV-infected sheep with pulmonary lesion and without pulmonary lesion analyzed by Mann Whitney two tail test.

1989; Eischen et al., 1991; Kreutz et al., 1992). Indeed, GM-CSF will increase surface antigen expression of CR3 and Fc γ IIR, phagocytic ability, cytotoxicity and TNF secretion of macrophages (Eischen et al., 1991). GM-CSF treated human MDM exhibit low grade proliferation (Elliott et al., 1989), but GM-CSF stimulates mouse MDM proliferation (Chen et al., 1988).

Other factors which are also present in serum are cytokines. IFN- γ is a very important cytokine which can activate macrophages and increase MHC class II DR, MHC class I and LFA-1 surface expression (Gonwa et al., 1986; Watanabe et al., 1991; Nash et al., 1992), but it restricts monocyte maturation (Andreesen et al., 1990). Another cytokine, TNF- α , acts as a potent stimulator of monocyte differentiation in myeloid leukemia cell lines, but TNF- α acts as an antagonist to IFN- γ to decrease MHC class II expression on mature monocytes (Watanabe et al., 1991, Gonwa et al., 1986).

In the experiments shown, monocytes were grown in high serum concentrations (10% FCS and 10% LS) to allow growth and differentiation of the monocytes *in vitro*. However, these serum concentrations were not enough to overcome the loss of MHC class II expression *in vitro*. A similar result in cultured human monocytes has been reported (Sztein et al., 1984). However, cultured human monocytes in teflon bags (suspension), in the presence of GM-CSF persistently express MHC class II DR for 2 weeks (Eischen et al., 1991) suggesting that adherence to plastic may down-regulate MHC class II expression. *In vivo*, activated T-helper cells secrete IL-2 which has been thought to be the primary stimulus causing IFN- γ release from immune T cells (Torres et al., 1982) which could then act on monocytes to allow MHC class II expression. *In vitro*, cultured MDM do not come into contact with activated lymphocytes. Con A-supernatant from activated lymphocytes used in this experiment may very well have contained IFN- γ which would explain the enhanced expression of MHC class II (both DR and DQ) molecules. But the Con A supernatant did not greatly increase MHC class I

and LFA-1 expression as is seen with human monocytes stimulated by recombinant IFN- γ (which causes an induction of specific mRNA) (Nash et al., 1992). The difference may be due to the presence of other factors within the Con A-supernatant acting against monocyte activation (in a similar manner to TNF- α) or the fact that the cultured ovine monocytes showed high levels of expression of MHC class I and LFA-1 which may be maximal without further stimulation.

Low levels of expression of CD4 molecules have been reported on human monocytes and cultured MDM (Collman et al., 1990) and support for its expression comes from the fact that CD4 acts as a receptor for HIV infection allowing infection of macrophage. (Kazazi et al., 1989; Collman et al., 1990; Crowe et al., 1992). However, expression of CD4 on sheep monocytes and cultured MDM has not been studied in depth. The experiments reported here show that fresh sheep monocytes and AM did not express CD4 molecules on their surface, but low intensity staining for CD4 molecules was seen after short term culture. The difference between CD4 expression on human and sheep monocytes is not understood, but may be a lower intensity of staining in the ovine system. However, internal and surface CD4 expressed on 7 day cultured MDM have been reported in humans (Collman et al., 1990). Perhaps, ovine monocytes contain internal CD4 and it and/or newly synthesized CD4 is expressed on the surface after culture. Moderate intensity staining for CD8 molecules was seen on MDM with similar kinetics of expression as CD4, but the biological significance of CD8 expression on MDM is not clear. A low percentage (10%) of fresh human monocytes expressing CD8 has been reported (Moebius, 1989). In addition, here, CD8 and MHC class II were inversely expressed on cultured MDM and Con A-supernatant activated MDM, but the reason for this is unknown. However, a similar inverse expression of MHC class II and CD14 on AM and peritoneal macrophages has been reported (Andreesen et al., 1990). Some molecules such as Fc γ RIII (CD16), α -chain of vitronectin (CD51), Max1 and Max3 are not expressed on fresh human monocytes, but are gradually

expressed on mature macrophages (Eischen et al., 1991) which is similar to the pattern of expression of VPM32 antigen and CD8 expression on cultured monocyte here.

4.3.2 Expression of surface antigens on MVV-infected MDM *in vitro*

Immature monocytes limit MVV replication (Narayan et al., 1983) and so monocyte cultures were not productively infected with MVV until they had differentiated. Three day cultured monocytes underwent phenotypic changes but did express VPM32 antigen, CD4 and CD8 and stable levels of MHC class I although MHC class II was still detectable. They were therefore considered to be macrophages and were used for infection with MVV. However, changes seen after infection were variable and so it was difficult to say whether they were caused by MVV infection, restriction of MVV infection or further differentiation of the cells with time in culture.

Seven-day cultured MDM have completely differentiated into macrophages (as defined by morphology, non-specific esterase staining and stable surface antigen expression). There was no significant difference in surface molecule expression on MVV infected and mock infected MDM up to 5 days post infection. However in some cultures there was enhanced expression of surface antigen early after viral infection. MDM may therefore increase their functional and biological activities in response to viral stimuli early in infection, but these changes were not further amplified by a cascade reaction due to the lack of lymphocytes in the cultures. The interaction of infected macrophages and lymphocytes may produce LV-IFN and enhance surface antigen expression as seen *in vivo* (Lairmore et al., 1988a; Kennedy et al., 1985). A similar phenomenon of enhanced antigen expression has been seen in AM infected with parainfluenza virus type 3 (Gonzalez, personal communication). There was no loss of surface antigen from MDM up to 5 days post infection with MVV (table 4.1), but by 7-8 days post infection degenerative

changes including a decrease in surface antigen expression was seen. This is probably due to productive replication of the virus as this is supported by the detection of viral antigen in the cytoplasm (figure 4.5). The kinetics of the changes also coincide with surface changes seen in MVV-infected MDM observed under transmission and scanning electron microscopy (chapter 3).

Fc receptors are important surface molecules for monocytes and macrophages. These receptors participate in phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and superoxide production (Anderson, 1989; Jungi et al., 1990; Sarmay, 1992). VPM32 is thought to be specific for Fc γ RIII (Gonzalez, 1988). MVV-infected MDM showed no decrease in the surface expression of VPM32 antigen 5 days postinfection.

In contrast, the HIV-infected (chronic) monocyte-like cell line (U937) shows decreased expression of CD4 (Mann et al., 1990a) and MHC class II, but increased expression of LFA-1 α (CD11a) and β (CD18) on the surface (Petit et al., 1987; Mann et al., 1990a). In HIV-infected MDM, HLA-DR increased, HLA-DQ decreased, and HLA-DP was unchanged (Mann et al., 1990a). A different study has shown that H9 cells after infection with simian immunodeficiency virus (SIV) *in vitro* (chronic infection) increase MHC class II antigen expression on their cell surface. This was caused directly by viral infection, not mediated by IFN- γ activity (Kannagi et al., 1987). The difference between results seen here and those with HIV and SIV may be due to the chronic (non lytic) infection seen with these two viruses, whilst MVV causes a lytic infection of MDM. Comparison to human T cells productively infected with HIV may be more real and these show decreased expression of CD2, CD3, CD4, CD8, CD11 and MHC class I (Stevenson et al., 1987; Scheppler et al., 1989).

4.3.3 Phenotypic expression on fresh AM from MVV-infected and control sheep

The results shown in this study are similar to those in previous reports, especially the increased expression of MHC class I and II on AM, and the decrease in CD4 : CD8 lymphocyte ratios in lungs from MVV-infected sheep (Cordier et al., 1992; Nash et al., 1992 ; Lujan et al., 1993). The increased amount of MHC class II on the AM surface in MVV-infected sheep may reflect local specific immune responses in the lung or just a local inflammatory reaction. Most studies report that MHC class II expression on monocytes and AM from HIV infected patients with AIDS is decreased compared to normal individuals, but unaltered in HIV infected patients with ARC (Heagy et al., 1984; Sei et al., 1986; Roy et al., 1987; Bray et al., 1993). This suggests that the decrease in the CD4 T cell population in AIDS patients may indirectly influence MHC class II expression on monocyte/macrophages perhaps through the action of IFN- γ . IFN- γ is found in the serum of HIV-infected patients which may help maintain or upregulate the MHC class II expression on circulating monocytes/AM from ARC patients (Roy et al., 1987). However, IFN- γ production in HIV-infected patients with AIDS is impaired (Murray et al., 1984), which may allow the downregulation of MHC class II expression on circulating monocytes of AIDS patients. A similar decrease in LFA-1 expression in monocytes is also seen in ARC and AIDS patients (Roy et al., 1987). In addition, increasing Fc γ RI expression on monocytes parallels elevated serum IFN- γ concentrations in HIV seropositive patients (Capsoni et al., 1992).

Although monocyte/macrophages are the predominant target cells of MVV the *in vivo* infection is not usually lytic but is persistent. *In situ* hybridization for viral RNA indicates that a large number of AM from MVV-infected sheep were infected (15% of AM, 2 weeks post infection) (Gendelman et al., 1985), but that viral replication in AM is strictly restricted, with only a small amount of virus being produced (Gendelman et al., 1985; Haase et al., 1986). This has been confirmed on

freshly prepared cytosmeears of BAL cells from naturally MVV-infected sheep in which 1-3 cells in 200- 300 cells were positive for *gag* p15 (data not shown). Therefore, the great increase in MHC class II molecule expression on AM may not be caused by virus infection in these cells. LV-IFN, known to be produced when lymphocytes are cultured with MVV-infected macrophages (Kennedy et al., 1985; Lairmore et al, Zink et al., 1989) may mediate the increase in AM class II expression as it has biological activities similar to IFN- γ (Narayan et al., 1985). In addition, it has been reported that there is a marked increase in the number of lymphocytes and neutrophils in interalveolar tissue and BAL in maedi (Cordier et al., 1992; Watt et al., 1992b; Lujan et al., 1993). These express more MHC class II (Cordier et al., 1992), but not CD5 antigen (Watt et al., 1992b) which suggests that these alveolar lymphocytes are activated. Therefore, the activated lymphocytes may release more cytokines as well as LV-IFN, which can enhance MHC class II expression on AM. A similar phenomenon is also seen on macrophages in MVV-induced arthritis (Kennedy-Stoskopf et al., 1989b; Harkiss et al., 1991).

Another important phenomenon noted in BAL cells from MVV-infected sheep with pulmonary lesions is a decrease in the CD4 : CD8 lymphocyte ratio. This is similar to the effect found in HIV infected patients with AIDS (Giorgi et al., 1989; Davidson et al., 1988). However, in HIV infected patients with ARC and AIDS, the change in CD4 : CD8 lymphocyte ratio is attributed to the depletion of the CD4⁺ subpopulation. In contrast, the decrease in CD4 : CD8 lymphocyte ratio in BAL cells from MVV-infected sheep does not result from a decrease in the CD4⁺ cell subpopulation, as CD4⁺ lymphocytes are not a target cell for MVV and most groups do not see a decreased CD4⁺ T cell population in the blood of MVV infected sheep. Cordier et al (1992) also reported a relative increase in the numbers of CD8⁺ lymphocytes in BAL fluid in naturally infected sheep, but not in experimentally infected sheep. Moreover, they found more CD4⁺ lymphocytes than CD8⁺ cells in lymphoid nodules in lungs of MVV-infected sheep (Cordier et al., 1992). Therefore

the decrease in CD4 : CD8 ratio may not only arise from an accumulation of the CD8⁺ lymphocyte subset, but may also be partially attributed to a redistribution of CD4⁺ lymphocytes between alveoli and nodules (Cordier et al., 1992). A decrease in the CD4 : CD8 lymphocyte ratio has also been reported in synovial fluid from joints with MVV induced arthritis (Kennedy-Stoskopf et al., 1989b; Harkiss, et al., 1991), but not in asymptomatic sheep (Lairmore et al., 1988) and in the CSF of MVV infected sheep (Torsteinsdottir et al., 1992). The increase in the CD8⁺ subpopulation in the lung may reflect an increase in cytotoxic activity of specific cells which eliminate MVV-infected AM. However, whether persistently infected AM can act as target cells for CTL's is still uncertain.

Increasing neutrophil chemotactic activity (NCA) and fibronectin release from AM has been reported in MVV-infected sheep (Cordier et al., 1990) which implies that some functional changes occur in AM of MVV-infected sheep. In this study the majority of experimentally infected sheep did not show gross lesions in their lungs. This may be due to their young age and short infective course when compared to naturally infected sheep. Moreover, the change in MHC class II expression on AM, CD4 : CD8 lymphocyte ratio or cell numbers in BAL coincides closely with pulmonary lesions. This suggests that the accumulation of CD4 and CD8 lymphocytes and neutrophils in BAL is initiated by a chemotactic activity released by MVV-infected AM (Cordier et al., 1990). Afterward, these activated lymphocytes may secrete IL-2 and IFN- γ or LV-IFN to activate AM and so increase MHC class II, class I and LFA-1 expression. This cycle may be repeated and gradually amplified many times during the long time course of the disease. A great number of activated macrophages and their products, in particular IL-1, TNF- α , and fibronectin (Cordier et al., 1990) accumulating in the lung may lead to interstitial pneumonia which interferes with normal pulmonary physiological functions.

4.4 Summary

Cultured sheep monocytes from blood induced the expression of some cell markers, such as CD4, CD8, and Fc γ RIII (VPM32 antigen) which are not expressed on fresh monocytes. These differentiated MDM also showed a greatly decreased MHC class II molecule expression after 3-5 days in culture. However, MHC class II could be re-expressed at the cell surface after treatment with Con A activated lymphocyte supernatant.

In vitro, early after MVV infection (first 3 days), most surface molecules including CD4, CD8, MHC class I and class II showed slightly enhanced expression, which may be similar to that seen on HIV persistently infected macrophages. Up to 5 days post infection MVV made no statistical difference to MDM surface antigen expression. But by 7 days post infection degenerative changes occurring due to the lytic nature of the infection caused generalized loss of surface antigen expression.

MHC class I, class II and LFA-1 expression on AM from MVV-infected sheep, especially those with maedi, were greatly enhanced compared to uninfected control sheep. Furthermore, the CD4 : CD8 lymphocyte ratio in BAL from MVV-infected sheep was greatly decreased.

CHAPTER 5

**PART A: *IN VITRO* STUDIES ON THE PHAGOCYTTIC ACTIVITY
OF CULTURED MONOCYTE-DERIVED-MACROPHAGES
INFECTED WITH MAEDI VISNA VIRUS**

**PART B: *STUDIES ON THE PHAGOCYTTIC ACTIVITY OF
MONOCYTES AND ALVEOLAR MACROPHAGES FROM MAEDI
VISNA VIRUS INFECTED SHEEP**

5.1 Introduction

Macrophages are programmed with a wide range of biological functions, predominantly to regulate the immune response of lymphoid cells (Harding et al., 1988; Weaver and Unanue, 1990; Unanue, 1992) and the nonspecific effector capacity of the body against invading microorganisms (Nathan, 1986). Macrophages exist throughout the body, but their functions are somewhat heterogeneous, depending on the microenvironment in which they are found (Brain, 1992). Chemotactic, phagocytic and microbicidal activities are basic functions of monocyte/macrophages migrating into inflammatory areas against invading microorganisms. Both Fc γ and C3b receptors on the cell surface of monocytes, macrophages and neutrophils mediate their phagocytic activity (Hed and Stendahl, 1982; Jungi et al., 1990; Sarmay, 1992). Therefore, analysis of Fc receptors and phagocytosis is commonly used to study the effector function of macrophages and their contribution to the pathogenesis of disease.

HIV induces the depletion of T lymphocytes which is a central feature of immunosuppression (Bowen et al., 1986; Fauci 1988; Lifson and Engleman, 1989; Geelen and Goudsmit, 1991). Monocytes expressing CD4 molecules also act as target cells for HIV infection and become a reservoir of viral persistence after infection (Lifson et al., 1989; Collman et al., 1990; Schmidmayerova et al., 1992) which may contribute to the immunosuppression seen during HIV infection. Defects in chemotaxis, phagocytosis and the bactericidal capacity of monocytes have been reported in HIV-infected patients with AIDS-related complex (ARC) or AIDS (Smith et al., 1984; Bowen et al., 1986; Wahl et al., 1989) although they vary from study to study. Loss of macrophage function may contribute to infection of AIDS patients suffering by opportunistic pathogens. However, functional defects in monocytes are usually not found in HIV-infected asymptomatic patients. Most functional defects in monocytes are attributed to the depletion of CD4⁺ T cells in AIDS patients (Bowen et al., 1986) rather than a direct defect at the monocyte level.

Clinically, immunosuppression in MVV-infected sheep is not a common feature (Georgsson et al., 1990) which suggests that macrophage function may not be defective during MVV infection. This may be because only a small proportion of macrophages are infected (Gendelman et al., 1985) or the fact that there is no depletion of CD4⁺ cells in MVV infection and so no loss of T cell help for macrophage function. However, in field studies, concurrent bacterial infections are often found in MVV-infected sheep with interstitial pneumonia. (Cutlip et al., 1977; Markson et al., 1983; Myer et al., 1988). *In vivo*, a decrease in the delayed type hypersensitivity response to PPD has been reported in MVV-infected sheep which is also thought to be a defect at monocyte level. (Dr. P. Bird, personal communication; Myer et al., 1988). However, the functional capabilities of monocytes and macrophages during MVV infections *in vivo* or *in vitro* are poorly documented.

Alveolar macrophages (AM) contribute to primary non-specific defense mechanisms against invading microorganisms in the lung. Many viral infections of the respiratory tract result in changes in the phagocytic and bactericidal activity of AM which may lead to concurrent bacterial infection (Fuentes, 1986; Boschert et al., 1988). Some functional activities of AM including fibronectin release and neutrophil chemotactic factor activity are activated during MVV infection (Cordier et al., 1990). LV-IFN has similar biological activities to IFN- γ (Narayan et al., 1985; Zink et al., 1987) and may be the cause of the above activation and enhanced MHC class II expression on AM (Kennedy et al., 1985; Cordier et al., 1990). This evidence suggests that the functional activity of AM in MVV-infected sheep may be enhanced. However superinfection with *Pasteurella spp* in maedi suggests the opposite, a defect in pulmonary defence mechanisms occurring in some MVV-infected sheep.

Results in the previous chapters have shown that cultured MDM infected with MVV do not show significant changes in surface molecule expression (chapter 4). This does not mean that effector functions are unaltered in these macrophages.

Therefore, the phagocytic function of monocyte/macrophages during MVV infection *in vitro* and *in vivo* was studied. The results clearly show that the phagocytic capacity of MVV-infected MDM, *in vitro*, was markedly decreased after 5 days postinfection, whilst the Fc receptor expression on MVV-infected MDM assayed by erythrocyte rosetting was not significantly changed. In contrast, the phagocytic activity of AM from MVV-infected sheep without lung lesions for opsonized bacteria is greatly enhanced, but that this activity may gradually become defective in MVV-infected sheep as pulmonary lesions develop.

5.2 Results

PART A: *In vitro* Phagocytic Capacity of MVV-Infected MDM

5.2.1 Kinetics of phagocytosis of particles by MDM

Using FITC-labelled particles as substrates for phagocytosis, it was possible to use flow cytometry to analyze phagocytic activity. The time of incubation and the concentration of FITC-labelled particles used in phagocytic assays were titrated in preliminary experiments. Most yeast and opsonized bacteria were bound and ingested by the MDM within 30 minutes at 37°C with only a slight increase with longer incubation times. Therefore a one hour assay was used. Phagocytosis of *P. hemolytica* by MDM was greatly affected by the bacterial concentration which was fed to the cultures (figure 5.4.A). High bacterial concentrations were toxic to the MDM which became granular and gradually detached from growing surfaces. Furthermore, opsonization was very important for phagocytosis of *P. hemolytica* and RBC. Different concentrations of antiserum also affected how well the opsonized particles were phagocytized (data not shown). In contrast, it was not necessary to opsonize yeast used in the phagocytic assay.

Figure 5.1 Phagocytosis of sheep RBC by cultured MDM

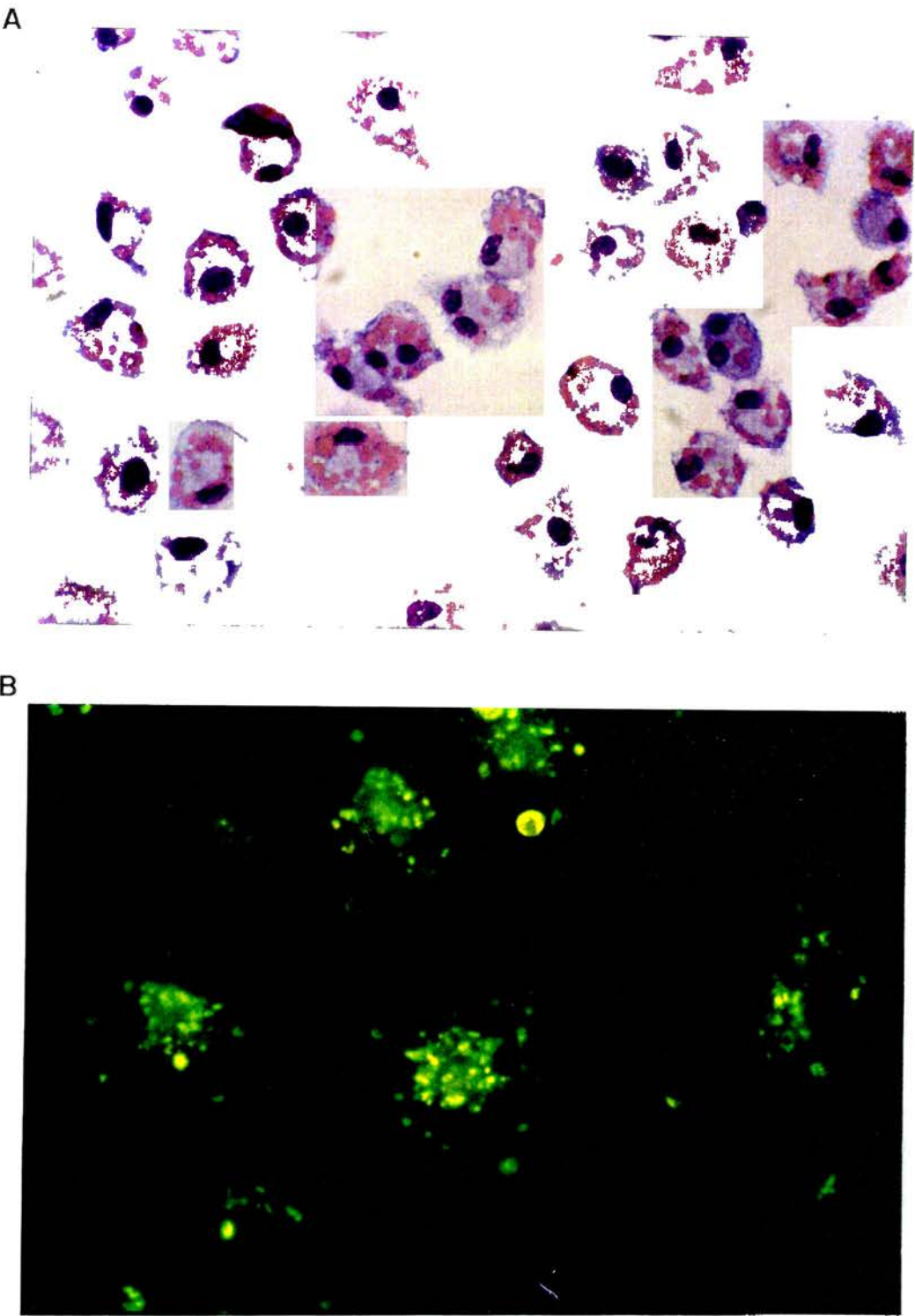
A. Sheep RBC were washed in PBS, opsonized with rabbit anti-SRBC antiserum (1 : 40) and then resuspended to 1% in RPMI/2%FCS medium as described in section 2.8.3 . Cultured MDM (day 12) were fed with 50 μ l of 1% opsonized SRBC in a final volume of 250 μ l RPMI/2%FCS medium for 1 hour at 37°C. Non-absorbed RBC were removed and external RBC were hypotonically lysed with distilled water for 30 seconds and then cells were immediately saturated by 2x PBS. Cells were fixed in methanol and then stained with Giemsa's solution and observed by a light microscopy.

Magnification: x500

B. As (A), except the RBC were pre-labelled with FITC in PBS (section 2.8.2) and then opsonized by rabbit anti-SRBC antiserum. MDM were observed by UV microscopy.

Magnification: x800

FIGURE 5.1



The fluorescence intensity of MDM was related to the number of FITC-particles which were bound to the cell surface or ingested by the cells. The phagocytic capacity of MDM was also directly examined by light or UV microscopy to compare with the fluorescence intensity detected by flow cytometry and actual particle ingestion. This showed that MDM could ingest up to 50 RBC per cell (figure 5.1 A & B) although the median of phagocytic activity was 16-25 RBC per cell.

5.2.2 Phagocytic activity of MVV-infected MDM for RBC

In order to study functional alterations in MDM after MVV infection, MDM were cultured in 24-well plates and the erythrocyte rosetting and phagocytosis of opsonized RBC were assayed by flow cytometry (figure 5.2). In the early phase of viral infection (before 5 days post infection), both MVV-infected and uninfected cells showed very high phagocytic activity, with nearly 90-95% of cultured MDM having erythrocyte rosetting and phagocytic activity (figure 5.2 and table 5.1). However, the phagocytic capacity of MVV-infected MDM by 5 and 7 days postinfection was dramatically decreased to 67% and 47% or less positive cells, respectively (figure 5.2 & table 5.1). There was a statistically significant difference between MVV-infected and mock-infected MDM at these time points ($p < 0.05$) (table 5.1). Experiments which analyzed FcR expressed on the surface of MDM in productive infection using erythrocyte rosetting did not show any difference between mock and MVV-infected MDM (figure 5.2).

5.2.3 Phagocytosis of yeast (*Candida utilis*)

Compared to RBC and *P. hemolytica*, yeast was directly and quickly ingested by MDM within 30 minutes. In a similar manner to RBC, a defect in phagocytosis of yeast by MVV-infected MDM was seen, in particular, after 5 days of virus infection (table 5.1). Although only 2 experiments were carried out and so

statistics cannot be applied, we believe that this difference is real. At 7 days post infection, incubation with yeast for 15 minutes gave the lowest percentage of cells with phagocytosed particles (figure 5.3). The number of cells positive for yeast then increased up to a 30 minute incubation but was unchanged after this. Therefore the defect in phagocytosis was not overcome by longer incubation times.

5.2.4 Phagocytosis of *P. hemolytica*

Phagocytosis of FITC-labelled and opsonized bacteria by MDM was examined to compare with phagocytosis of RBC and yeast. There was no impairment of phagocytosis of opsonized bacteria by MDM infected with MVV after 5 and 7 days postinfection (table 5.1 and figure 5.4.B). However, the fluorescence levels indicated may not be completely due to internalized bacteria as many externally bound bacteria were detected by UV microscopy (data not shown). Unlike RBC these externally bound particles could not be lysed by water. Attempts to quench external fluorescence by treatment with crystal violet failed as this also decreased the internal fluorescence intensity and so made the assay less sensitive (data not shown). It is therefore likely that any fall in phagocytic activity for opsonized bacteria may be masked by the steady FcR levels already shown by erythrocyte rosetting.

Figure 5.2 Comparison of phagocytosis and erythrocyte rosetting of opsonized RBC by MVV- or mock-infected MDM

Phagocytosis: MDM were cultured and infected with MVV, 1 TCID₅₀ per cell, in 24-well plates on day 5, 7 and 9 respectively. Fifty µl of 1% FITC-labelled and opsonized RBC in RPMI/2%FCS were fed to the culture for 1 hour. After washing, external RBC were lysed with distilled water and then saturated with 2x PBS. MDM were harvested and transferred to Rhesus tubes. After centrifugation 5,000 MDM were analyzed by flow cytometry.

Erythrocyte rosetting: MDM were harvested, washed in PBSA with 0.01% sodium azide and then pelleted in Rhesus tubes. Fifty µl of 1% opsonized SRBC were added to each Rhesus tube and cells incubated on ice for 30 minutes. Cells were resuspended in PBSA and immediately analyzed by flow cytometry.

This is a representative profile of 3 separate experiments comparing the phagocytic and erythrocyte rosetting activity of MVV-infected and uninfected MDM. The unshaded areas (□) are negative control MDM without RBC and the shaded areas (■) are MDM binding or ingesting RBC. Erythrocyte rosetting (upper panel) was compared to phagocytosis (lower panel). Figures on the top right of panel are the mean fluorescence intensity of RBC fed MDM.

FIGURE 5.2

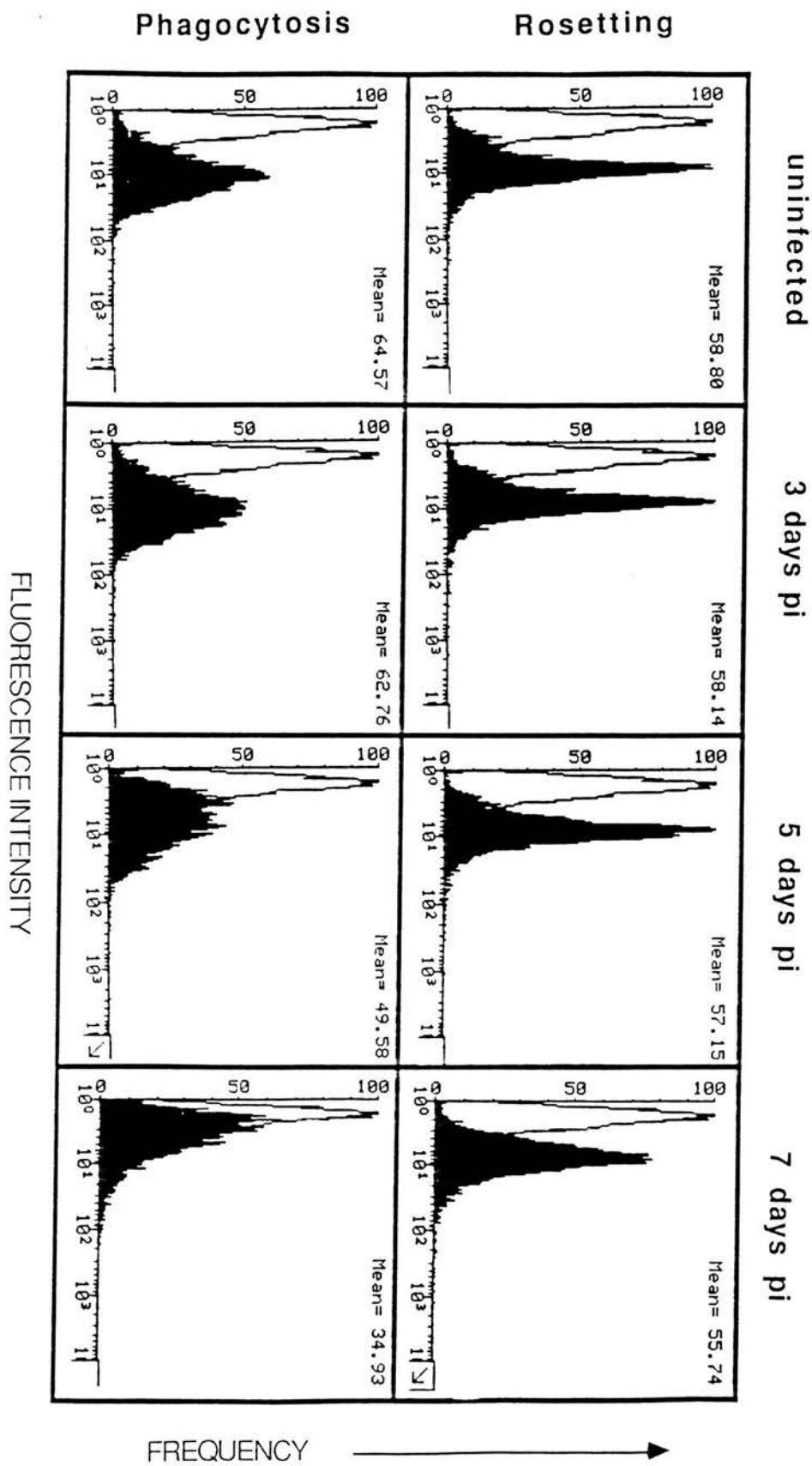


Figure 5.3 Phagocytosis of yeast by MDM

MDM were cultured and infected with MVV, 1 TCID₅₀ per cell, in 24-well plates on day 5, 7 and 9 respectively. Yeast (*Candida utili*) were cultured and labelled with FITC as described in section 2.8.2. After 3 washes, the yeast were resuspended at 1x10⁸/ml in RPMI/2%FCS. 50µl of FITC-labelled yeast in 250µl RPMI was added to MDM either infected or mock infected with MVV. After 15, 30, and 60 minutes incubation at 37°C, cells were washed three times with PBS. Cells were removed from the plates, fixed in 1% paraformaldehyde in PBS for 1 hour, and then analyzed by flow cytometry.

The unshaded area (□) indicates negative control, uninfected MDM which had not been fed FITC-labelled yeast. The shaded area (■) shows MVV-infected or uninfected MDM fed with FITC-labelled yeast. Data are expressed as percentage positive cells using a 1% gate from the control.

FIGURE 5.3

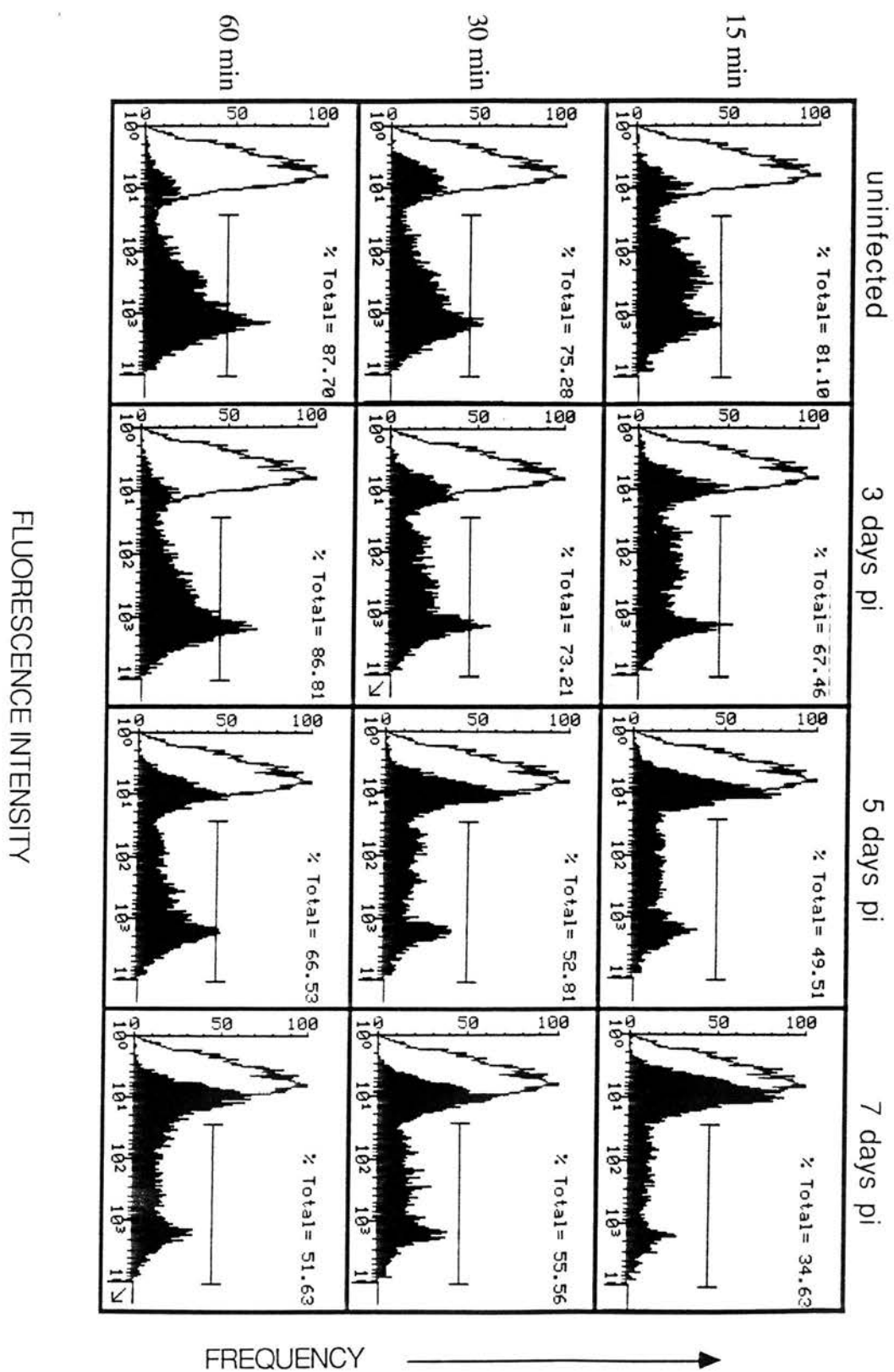


Figure 5.4 Phagocytosis of opsonized *P. hemolytica* by MDM

MDM were cultured and infected with MVV, 1 TCID₅₀ per cell, on day 7, 9 and 11. *P. hemolytica* was cultured on blood agar at 37°C overnight. Bacterial colonies were removed and resuspended in PBS. The bacterial concentration was adjusted to OD₅₅₀=0.5 in PBS (around 3-4 x10⁸ CFU/ml), labelled with FITC, and opsonized by rabbit anti-*P. hemolytica* antiserum (1:10) as described in section 2.8.2. After two washes, the bacteria were resuspended to the original concentration in RPMI medium and used in the assay. Phagocytosis was assayed as previously described in section 2.8.4.

A. Titration of bacterial concentration in the phagocytosis assay. Unshaded areas (□) indicate cultured MDM fed with 10µl (1), 50µl (2) and 100µl (3) of the standard bacterial suspension in 250µl RPMI medium at 37°C for 1 hour. The shaded area (■) shows the background control.

B. A representative profile from 3 separate experiments of bacterial phagocytosis by infected MDM. Fifty µl of bacterial suspension in 250µl RPMI/2%FCS was used in this assay. The shaded area (■) shows the negative control MDM overlapped by an unshaded area (□) which was MDM fed with FITC-labelled bacteria. Numbers at the top right of panels show the percentage positive cells compared to a 1% gate set on negative cells.

FIGURE 5.4

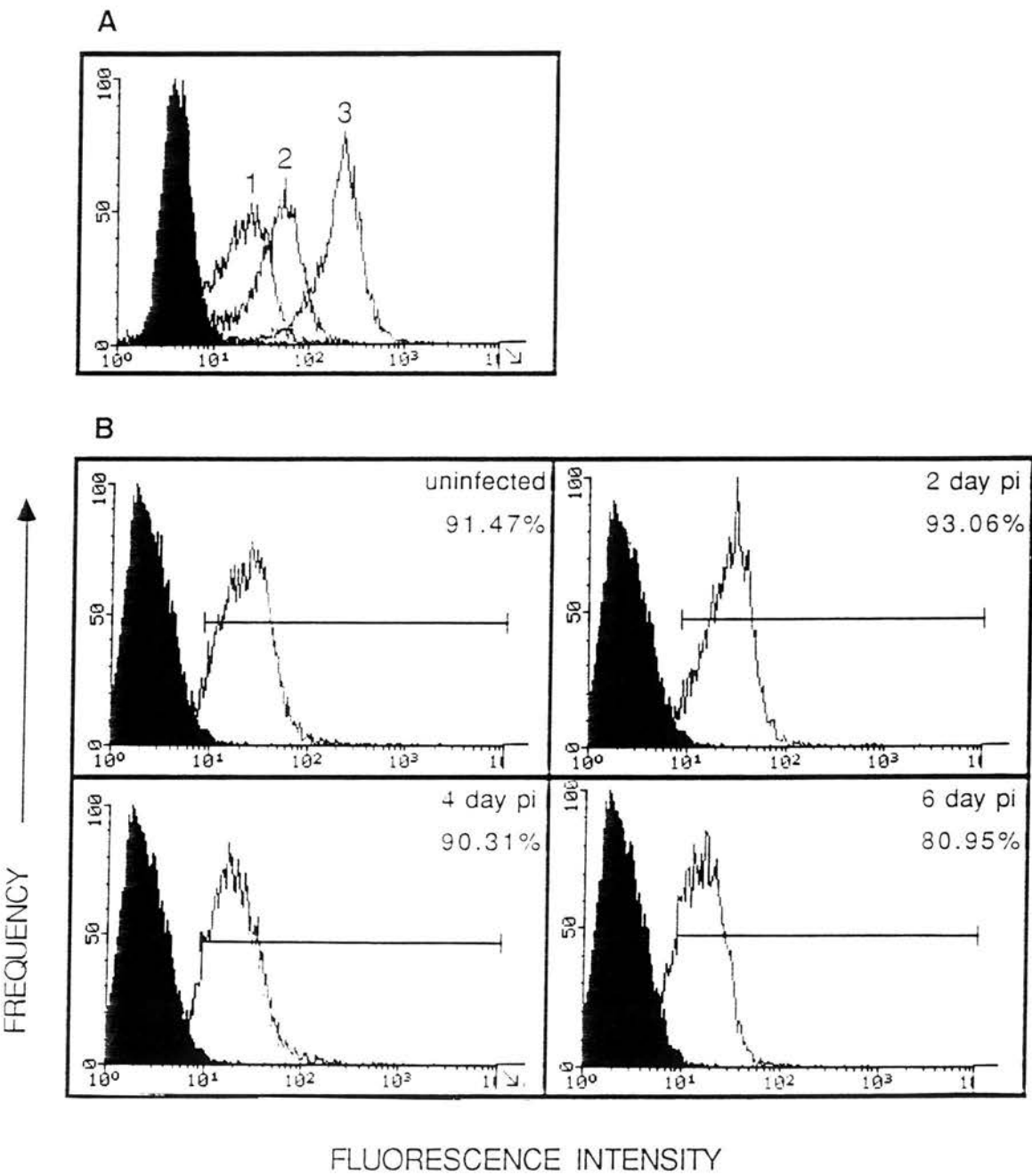


TABLE 5.1 Phagocytic Activity of Cultured Monocyte-derived Macrophages Infected with Maedi Visna Virus

| Target particles | uninfected | post infection** | | |
|---|-----------------|------------------|------------------------------|------------------------------|
| | | 3 days | 5 days | 7 days |
| RBC (N=5) | 95.7 \pm 3.0* | 95.2 \pm 3.7 | 66.9 \pm 14.0 ^a | 47.4 \pm 11.0 ^a |
| Yeast (N=2) | 88.5 \pm 1.2 | 87.1 \pm 0.3 | 71.7 \pm 7.3 | 49.0 \pm 9.4 |
| <i>P. hemolytica</i> (N=3) ⁺ | 89.2 \pm 3.0 | 91.3 \pm 1.6 | 87.8 \pm 3.0 | 87.3 \pm 3.6 |

* Data are expressed as percentages of positive cells with phagocytic activity by setting a 1% gate on negative controls.

** Cultured MDM on day 5, 7 and 9 were infected with MVV, 1 TCID₅₀ per cell, and phagocytic activity (60 minute incubation) was analyzed on day 12 by flow cytometry.

+ Bound and ingested bacteria.

a Indicates a statistically significant difference (P < 0.05) between uninfected and MVV-infected MDM using a Mann Whitney two tail test.

Part B: Phagocytic and Erythrocyte Rosetting Activity of Monocytes and AM from MVV-Infected Sheep

5.2.5 Phagocytosis and erythrocyte rosetting by fresh monocytes from MVV-infected sheep

The phagocytic capacity of fresh monocytes during MVV infection was studied using a modified double staining method and flow cytometry. VPM 65 is a monoclonal antibody specific for CD14 which is highly expressed on monocytes (but expressed with low density on DC and neutrophils) and it was used to label monocytes in total PBMC. The phagocytic and erythrocyte rosetting activity of lymphocytes stained with SBU-T4, SBU-T8, VPM8, and ST197 were used as controls to compare with the monocyte activity. The results clearly reveal that large B and T lymphocytes (gated in monocyte enriched area) had a subpopulation expressing FcR which bound the opsonized RBC and bacteria, but the cells did not ingest RBC (figure 5.5). In contrast, a phagocytic activity was clearly found in fresh monocytes which were labelled with VPM65 (figure 5.5). Compared to cultured MDM, fresh monocytes did not show a very strong phagocytic capacity for opsonized RBC and bacteria. The activities greatly varied in individual sheep.

Nine experimentally infected sheep (2-3 years old) without clinical symptoms, five naturally infected sheep from the field, and eight non infected control sheep (2-3 years old) were used to investigate any changes in the phagocytic function of fresh monocytes. There was no difference in the erythrocyte rosetting activity of monocytes from uninfected or MVV infected sheep (figure 5.6). There was also no difference in the phagocytic activity of monocytes from these sheep for RBC or *P. hemolytica* (figure 5.6). All groups were analyzed using a Mann Whitney non-parametric test.

Figure 5.5 Phagocytic and erythrocyte rosetting activity of PBMC

FITC-labelled and opsonized RBC and *P. hemolytica* were prepared as described in the materials and methods (section 2.8.2 & 2.8.3). One $\times 10^6$ PBMC were pelleted in Rhesus tubes and then fed with 50 μ l FITC-targets. The cell mixture was incubated at 37°C for 1 hour. External RBC were removed by hypotonical lysis by distilled water for 30 seconds which was then neutralized with 2x PBS followed by 3 washes with PBSA. The cells were stained with monoclonal antibodies followed by anti-mouse IgG-phycoerythrin conjugate (section 2.7.2). After 3 washes with PBSA, cells were fixed in 1% paraformaldehyde and 10,000 cells gated in the monocyte enriched area were analyzed by flow cytometry. For the erythrocyte rosetting assay, PBMC were first stained with the monoclonal antibodies followed by the phycoerythrin conjugate and then mixed with opsonized RBC on ice for 30 minutes. PBMC only, PBMC fed with FITC-labelled particles and PBMC stained with Monoclonal antibody were used as background controls.

These are representative profiles of B and T cells (sheep 848A) and monocytes (sheep 1235T) mediating the phagocytic and erythrocyte rosetting activity in PBMC. Panels (row 1 to 5) were PBMC stained with SBU-T4 (anti-CD4), SBU-T8 (anti-CD8), ST-197 (anti-T19), VPM8 (anti-Ig light chain) and VPM65 (anti-CD14) respectively, against FITC-labelled *P. hemolytica* and RBC phagocytosis and RBC rosetting.

FIGURE 5.5

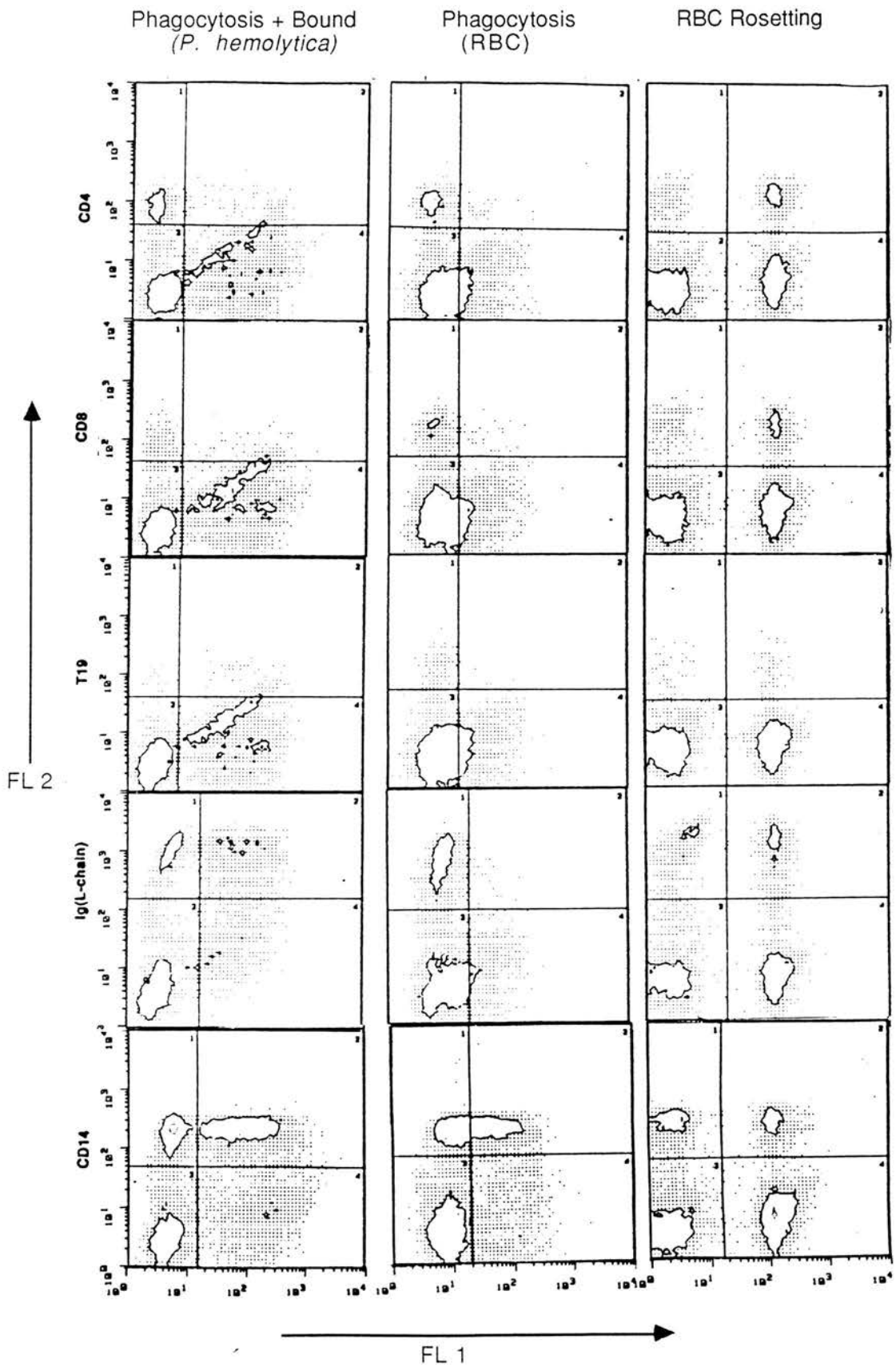
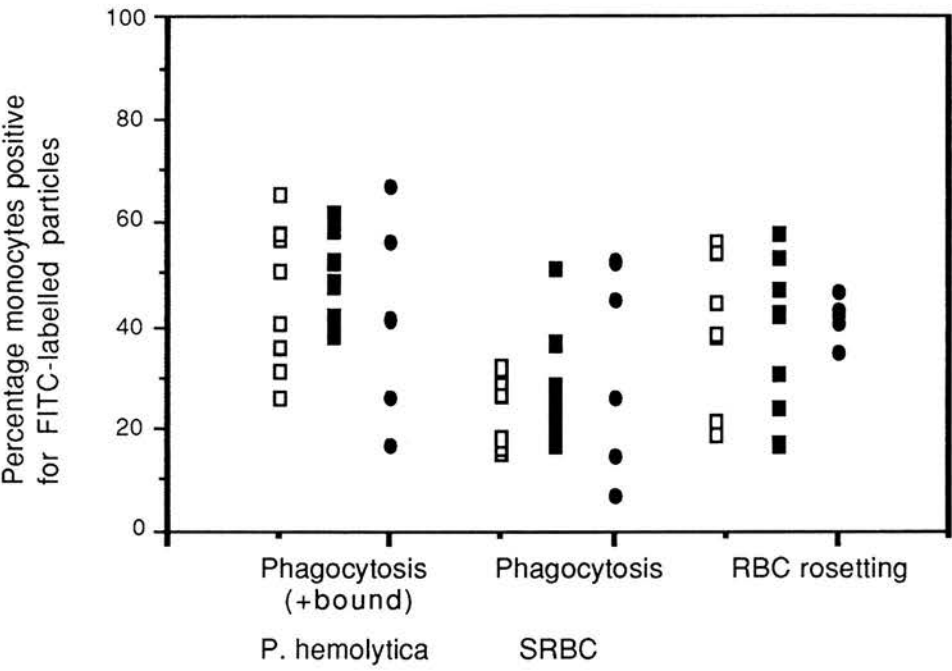


Figure 5.6 Comparison of the phagocytic and erythrocyte rosetting activity of monocytes from MVV-infected and uninfected control sheep

PBMC from 8 control sheep (□), 9 experimentally infected sheep without clinical symptoms (■) and 5 naturally infected sheep from the field (●) were used in this assay. Phagocytosis and erythrocyte rosetting by monocytes were assayed by the modified double staining method as described in section 2.8.7. Ten thousand cells were analyzed in the monocyte enriched area by flow cytometry. Data are expressed as the percentage of VPM65 positive cells (monocytes) with phagocytic activity.

FIGURE 5.6



5.2.6 Phagocytosis and erythrocyte rosetting by AM from MVV-infected sheep

Five experimentally and eight naturally MVV-infected sheep were used in this e-experiment. Eight grossly normal lungs collected from control animals or the abattoir were used as controls. The control sheep were not age and breed matched to the MVV-infected sheep. A direct phagocytic assay detected by flow cytometry was used (see section 2.8.6).

A representative profile of phagocytosis and erythrocyte rosetting by AM is shown in figure 5.7. There was no significant difference in the erythrocyte rosetting or phagocytosis activity for RBC in any of the sheep groups (table 5.2). However, with *P. hemolytica* the phagocytic activity of AM was enhanced in MVV infected sheep without lung lesions (MF 46.7 increased to 70.8). This difference was statistically significant ($P=0.0481$). There was no difference in phagocytosis of *P. hemolytica* between the control group and AM from sheep with MVV induced lung lesions (table 5.2)

5.2.7 Phagocytic capacity of the monocyte/macrophage lineage.

The phagocytic capacity of fresh monocytes and AM taken from normal sheep and uninfected MDM (day 12) in the previous studies are summarized and shown in figure 5.8. Cultured MDM had a very strong phagocytic and erythrocyte rosetting activity. In contrast, AM also had a strong phagocytic activity, but up to 45% of AM from control sheep did not show erythrocyte rosetting activity as detected by flow cytometry (table 5.2). Fresh monocytes showed the lowest phagocytic capacity when compared to AM and MDM. Erythrocyte rosetting by fresh monocytes gave 35% positive cells in normal control sheep (figure 5.6).

Figure 5.7 Phagocytosis and erythrocyte rosetting by AM

FITC-labelled and opsonized targets were prepared as described in materials and methods (section 2.8.2 & 2.8.3). 1×10^5 AM were pelleted in Rhesus tube and then mixed with 50 μ l of FITC-labelled targets. Cells were incubated for 1 hour at 37°C. External RBC were hypotonically lysed with distilled water for 30 seconds and then saturated with 2x PBS. After 3 washes, cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry. In the erythrocyte rosetting assay, AM were washed with PBSA and then mixed with 50 μ l FITC-labelled, opsonized RBC at 0°C for 30 minutes and immediately analyzed by flow cytometry.

These are representative profiles of phagocytic and erythrocyte rosetting assays by AM from a MVV infected sheep. The unshaded area (\square) indicates the negative control cells which were not fed targets and are overlapped by a shaded area (\blacksquare) which was AM fed with the FITC-labelled targets. Mean fluorescence intensity was used to express the phagocytic activity with *P. hemolytica* and RBC. Due to the presence of 2 distinct cell populations, percentage positive cell number was used for erythrocyte rosetting with a 1% positive region set on negative control cells.

FIGURE 5.7

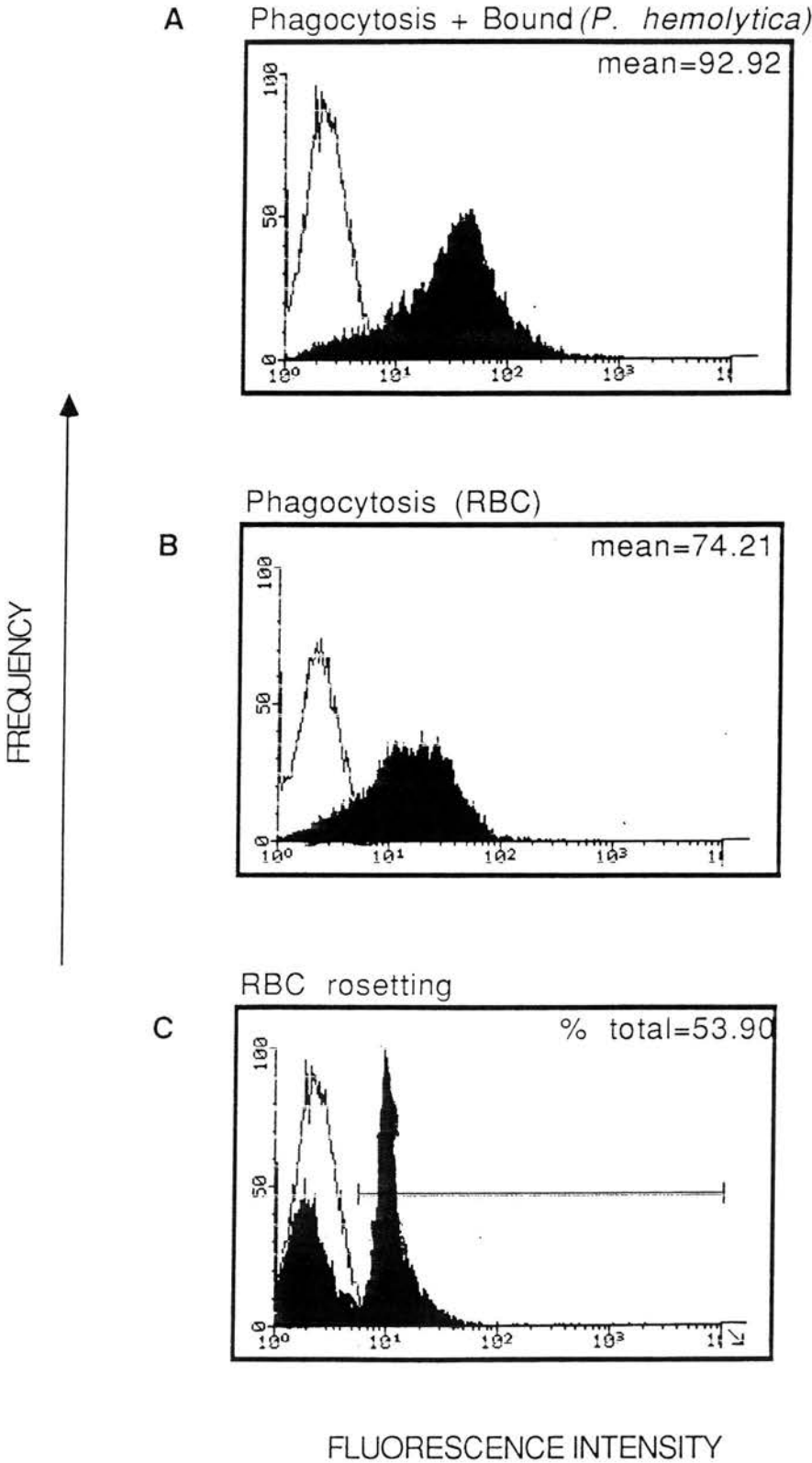


Figure 5.8 Comparison of the phagocytic and erythrocyte rosetting activity of monocytes, AM, and cultured MDM

This figure shows the summarized results from the previous experiments comparing the phagocytic and erythrocyte rosetting activity by monocytes, AM and cultured MDM from uninfected control sheep or uninfected MDM (day 12). Data are expressed as the percentage positive cells from a 1% positive region set on the negative control with standard deviations shown.

FIGURE 5.8

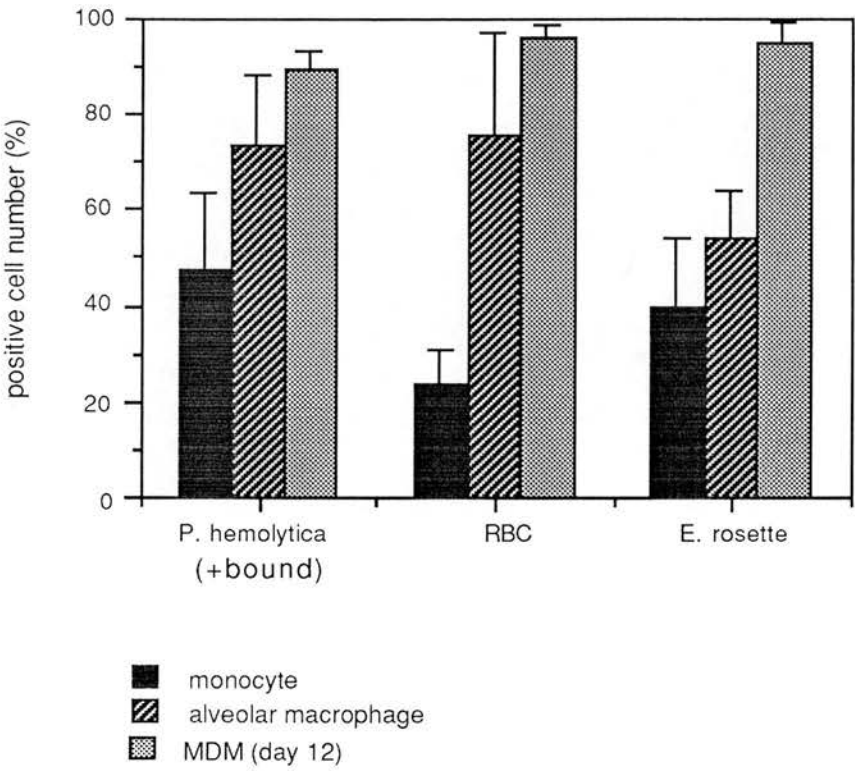


TABLE 5.2 The Comparison of Phagocytic and Erythrocyte Rosetting Activity by Alveolar Macrophages from MVV-Infected and Control Sheep

| Group | Phagocytosis + bound (<i>P. hemolytica</i>) MF | Phagocytosis (RBC) MF | Erythrocyte rosette % |
|---|---|-----------------------------|-----------------------------|
| Control sheep (N=8) | 46.7 ± 12.4* | 54.4 ± 13.7 | 54.5 ± 9.8 |
| MVV-infected sheep without lung lesions (N=5) | 70.8 ± 20.0 ^a (P=0.048)** | 54.2 ± 6.1 (P=0.510) | 50.8 ± 6.4 (P=0.671) |
| MVV-infected sheep with lung lesions (N=8) | 41.8 ± 16.1 (P=0.495) | 47.1 ± 14.5 (P=0.318) | 48.1 ± 7.5 (P=0.220) |

* Data are expressed as mean fluorescence intensity (MF) (MF with FITC targets - MF without FITC targets) or percentage of positive cells by setting a 1% gate on the negative control.

** P value between MVV-infected and control sheep using a Mann Whitney two-tail test.
(a) indicates a statistically significant difference.

5.3 Discussion

Different approaches to determining phagocytosis and Fc receptor expression have been developed. In general, the assay most frequently used to quantify phagocytosis directly counts the number of cells containing ingested particles under a microscope (Boschert et al., 1988; Washburn et al., 1985). However, this procedure is very tedious and time-consuming, and is subject to operator error due to personal perception and the accuracy of counting large numbers of cells by eye. More automated assays include measurement of ingested RBC by a colorimetric assay using haemoglobin as the substrate (Jungi et al., 1990); the total fluorescence intensity of ingested FITC-labelled particles in lysates of the phagocytic cells detected by spectrofluorimetry (Oda et al., 1986; Ragsdale et al., 1989); and radioactive counting of $^{51}\text{Chromium}$ -labelled particles instead of FITC-labelled particles are all employed in phagocytic assays (Salmon et al., 1984). However, these methods have some limitations which include: equivalence of the phagocytic cell number in parallel wells, harvesting errors and labelling levels with radiation as well as operator safety.

In initial studies, the different approaches mentioned above were tested and compared. Flow cytometry (Bjerknes et al., 1989; Buschmann et al., 1989, Tuijnman et al., 1990) was the best and simplest approach for the phagocytic assays used in this study. It can not only quantitatively analyze the number of cells with phagocytic activity, but can also qualitatively analyze the number of ingested RBC in each phagocyte by fluorescence intensity. Moreover, it can count more than 5,000 cells within specific parameters in a couple of minutes. Therefore, it is a good and convenient method to approach the phagocytic assay.

Compared to yeast and bacteria, RBC were the best targets in phagocytic assays, as external RBC can easily be lysed with distilled water. The assay then only measures those cells which have internalized RBC. Bacteria and yeast cannot be removed from the surface by this method and so there are still some labelled

particles binding to the cell surface. Therefore results with yeast and bacteria are the sum of surface binding and internalization. When crystal violet was used to quench external fluorescence (Hed et al., 1982), it was found that all fluorescence was interfered with and so this method could not be used in flow cytometry. A method for stripping the external particles from the cell surface is therefore necessary (eg protein digestion) before surface binding and internalization of yeast and bacteria can be separated. However, treatment with trypsin to remove externally bound *P. hemolytica* was not successful in this study (data not shown).

Monocytes and macrophages have several distinct lectin-like receptors for sugar residues which are important in the phagocytosis of bacteria and fungi (Gordon and Mokoena, 1989). *P. hemolytica* (Gram negative bacteria) contains an outer lipid bilayer which may inhibit direct binding to macrophages (Maheswaran et al., 1980) therefore opsonization was used. The mannosyl fucosyl receptors (MFR) and the receptor for β -glucan on the surface of monocytes and macrophages are involved in receptor-mediated phagocytosis of yeast and *Leishmania* (Blackwell et al., 1985; Czop and Austen, 1985). Hence, it is not necessary to opsonize yeast with antiserum or complement to mediate phagocytic activity. However, yeast which have been opsonized with serum show greatly enhanced internalization (Oda et al., 1986). This means that complement receptors, Fc receptors and MFR of monocytes and macrophages collaborate in the binding and phagocytosis of yeast and bacteria.

Both Fc γ and C3b receptors are very important surface molecules which mediate phagocytosis on macrophages and neutrophils (Sarmay, 1992; Jungi et al., 1990; Anderson, 1989; Hed and Stendahl, 1982). The C3b receptor primarily promotes attachment so that the particle is bound onto the membrane, whereas the FcR mediates ingestion (Hed and Stendahl et al., 1982). The ingested microorganisms in phagosomes may be killed by the peroxidase-hydrogen peroxidase system (Locksley et al., 1987; Nakagawara et al., 1981). Internalized particles may also be partially degraded and peptides derived can become associated

with MHC class II molecules. Antigen breakdown and re-expression on the surface of macrophages plays a major role in initiating an immune response (Brodsky and Guagliardi, 1991).

After culture, Fc γ R expression on MDM and their phagocytic activity are greatly increased (Jungi et al., 1986) and the experiments shown here agree with these findings. Modulation of the Fc γ receptor associated with a functional phagocytic impairment has been reported (Fleit et al., 1982). Here FcR binding of opsonized RBC was unimpaired in MDM which had been infected with MVV for more than 5 days. However there was a decrease in ingestion of these bound RBC at these times post infection. Therefore, productively MVV infected MDM had a functional impairment in the macrophage not reflected in the apparently normal phenotype and surface particle binding at these late times post infection.

When intracellular killing of bacteria was assayed in cultured MDM either uninfected or MVV infected, the activity was found to be very low (10-30% bacteria killed if fed to MDM at 1-2 bacteria per MDM) and variable from experiment to experiment (data not shown). However, ConA supernatant treatment increased this activity so that greater than 90% of bacteria were killed within 1 hour. In other systems the intracellular killing activity of MDM is also low if compared to the bactericidal activity of peritoneal or alveolar macrophages (Maheswaran et al., 1980; Collins et al., 1983). It is thought that the peritoneal or alveolar macrophage activity is similar to that found *in vivo*. *In vitro* the differentiation of monocytes into macrophages is accompanied by an apparent reduction in the ability of cultured MDM to produce H₂O₂ and O₂⁻ (Nakagawara et al., 1981). Phagocytosis of an organism does not always activate the killing mechanism, but killing can be amplified by the treatment of macrophages with LPS, TNF, GM-CSF and IFN- γ (Murray et al., 1987; Weiser et al., 1987; Bermudez and Young, 1988) suggesting cytokines present in local inflammatory areas or regional lymph nodes during an acute immune response *in vivo* are very important in the upregulation of

microbicidal activity of macrophages, but that these cytokines are not present *in vitro* cultures.

There was no defect in the binding and phagocytic activity of monocytes from MVV infected sheep for *P. hemolytica* in this study. This suggests that the low level of MVV in monocytes from infected sheep does not interfere with their function. In addition, there may be no build up of infected monocytes in the blood as circulating monocytes quickly enter into tissues and differentiate into macrophages (Beelen et al., 1989). A decrease in the phagocytic and bactericidal capacity of monocytes from HIV-infected patients, especially ARC and AIDS patients (Bravo-Cuellar et al., 1992; Szkaradkiewicz et al., 1992) and from bovine immunodeficiency virus (BIV)-infected calves (Onuma et al., 1992) has been reported. Bender et al (1988) have reported a defect *in vivo* in the clearance of particles injected into the blood stream by monocytes from AIDS patients. However, Eales et al (1987) have also reported that there is no defect in the microbicidal activity of monocytes for *Candida*, *Aspergillus*, *Toxoplasma* and *Chlamydia* in AIDS (Eales et al., 1987; Murry et al., 1987). The differences in these observations may come from differences in the depletion of CD4 lymphocytes and the impairment of cytokines released by T lymphocytes in AIDS patients which may regulate monocyte function *in vivo*. Also HIV-infected patients receive different immunological and/or drug therapies which may affect monocyte function. There is a defect in chemotaxis of monocytes in HIV infected patients with AIDS, especially in those patients with Kaposi's sarcoma or opportunistic infections (Martin et al., 1988; Smith et al., 1984; Poli et al., 1985a; Wahl et al., 1989). This evidence suggests that an impairment in phagocytic activity or intracellular killing ability by monocytes does not occur in early HIV infection, but is seen in patients in the late stages of disease (AIDS) and this impairment may contribute to the secondary infections and neoplasms which develop in late HIV disease.

It has been reported that alveolar lymphocytes in sites of lesions during MVV infection are in an activated state (Watt et al., 1992b; Cordier et al., 1992) and it is assumed that cytokines from activated lymphocytes are released into the blood stream or local tissues where they may enhance macrophage functions. Here, binding and phagocytosis of *P. hemolytica* by AM from MVV infected sheep without pulmonary lesions was greatly enhanced when compared to that from control sheep, but phagocytosis of RBC by AM was not. This difference may be due to external bound *P. hemolytica* rather than the phagocytized particles. The difference in particle size (RBC > bacteria) may also contribute. Erythrocyte rosetting was unaffected in the AM from these animals suggesting that any difference may be due to the sugar residue receptors of AM. There was no difference in the phagocytic and erythrocyte rosetting activity of AM from sheep with lung lesions compared to controls. The groups were not age matched and limited numbers were used which may have been why significant change were not seen.

Pasteurella hemolytica is an important opportunistic pathogen of the upper respiratory tract of sheep. Pasteurellosis is generally associated with various forms of stress including fatigue or viral infections. The disease is commonly found in MVV-infected sheep with interstitial pneumonia (Markson et al., 1984; Myer et al., 1988). However the data presented here suggest that early events in AM interaction with tissue bacteria (binding and internalization) are not affected in sheep with MVV induced lung lesions. Therefore later stages in the anti-bacterial function of AM (eg intracellular killing) or other factors involved in normal pulmonary function must be reduced in sheep with pasteurellosis. Normal pulmonary immunity also involves local immunity (CMI, IgG, IgA), pulmonary secretions and physiological conditions in the lung (Christensen and Mousing, 1992; Burrells, 1985). Perhaps a major contribution is the alveolar wall thickening seen in lungs affected by maedi which reduces transduction of plasma components into respiratory system. This

may lead to a failure in opsonization and allow bacteria entering the lower respiratory tract to replicate.

5.4 Summary

The phagocytic and erythrocyte rosetting activities of monocyte/macrophages from different organs were heterogeneous. Cultured MDM displayed the strongest phagocytic capacity and erythrocyte rosetting activity when compared to fresh monocytes and AM.

In vitro, the phagocytic capacity of MVV-infected MDM for RBC and yeast was defective by 5 days postinfection, but not earlier in infection.

There was no significant difference in phagocytic capacity and erythrocyte rosetting activity by fresh monocytes from MVV-infected and uninfected control sheep. In contrast, the binding and phagocytosis of *P. hemolytica* by AM from MVV-infected sheep without pulmonary lesions was greatly increased compared to AM from uninfected sheep. No defects were seen in the binding and phagocytosis of *P. hemolytica* or RBC by AM from sheep with pulmonary lesions.

CHAPTER 6

THE EFFECT OF MAEDI VISNA VIRUS INFECTION ON THE ANTIGEN PRESENTING FUNCTION OF MACROPHAGES

6.1 Introduction

The immune response involves a complex interaction between T-lymphocytes and antigen presenting cells (Weave and Unanue, 1990). Initiation of the immune response is also regulated by a complex cytokine network released by the activated T lymphocytes and antigen presenting cells (APC) (Weaver et al., 1988 & 1990). $CD4^+$ T-cells recognize antigen associated with self MHC class II molecules, whilst $CD8^+$ T-cells see antigen on MHC class I molecules (Unanue, 1992). Although T-lymphocyte activation is at the center of the immune response, APC play an important role in antigen processing and presentation (Ziegler and Unanue, 1981).

The dysfunction of the immune response in HIV infection, including the impairment of proliferative responses to mitogens, recall antigens, T-cell mediated cytotoxicity and natural killer cell mediated lysis has been well discussed (Bowen et al., 1986). Immunosuppression in HIV-infected patients with AIDS is believed to be a result of the depletion of $CD4^+$ lymphocytes and impairment of lymphokine production (Levy, 1993; Bowen et al., 1986; Fauci, 1988; Lifson and Engleman, 1989). However in late stages of infection dendritic cells, follicular dendritic cells and Langerhan's cells in the spleen, lymph nodes and skin are also gradually destroyed (Fox and Cottler-Fox, 1992; Eales et al., 1988; Tschachler et al., 1987; Cameron et al., 1987; Macatonia et al., 1989; Armstrong and Horne, 1984) which contributes to the immunological abnormalities in HIV infection. Monocytes also act as target cells for HIV infection and this cell type becomes a reservoir of HIV infection (Schmidtayerova et al., 1992; Schuitemaker et al., 1992a; Gendelman et al., 1985; Ho et al., 1986). Hence, the HIV/macrophage interaction is closely related to disease progression, especially during the asymptomatic stage of HIV disease.

It has been reported that the accessory cell function of monocytes in early HIV infection is not significantly affected (Terpstra et al., 1989; Clerici et al.,

1990), but other researchers have presented different results which show a decrease in antigen presenting and accessory cell functions of monocytes for T-cells in HIV-infected patients before the appearance of other symptoms of AIDS (Petit et al., 1988; Ennen et al., 1990; Melendez-Guerrero et al., 1991). The monocyte cell line, U937, and macrophage hybridomas also reduce their MHC class II expression (Petit et al., 1987; Sperber et al., 1993) and accessory cell function for anti-CD3 monoclonal antibody induced T-cell lymphoproliferative responses after HIV infection (Petit et al., 1988). This suggests that HIV infection in macrophages may contribute to immunopathological features observed in early HIV infection and in AIDS patients. Addressing antigen presenting function of monocyte/macrophages in HIV infection *in vivo* is very complex, because many functional defects of monocytes and macrophages in HIV infection may be related to the depletion of CD4 lymphocytes rather than direct virus infection. However, in MVV infection there is no depletion of lymphocytes and so the effect of lentivirus/macrophage interaction on antigen presenting function may be studied in this disease.

In MVV infection, macrophages not only act as predominant target cells for virus, but also present viral antigens to induce immune responses. The development of humoral and cellular immune responses to MVV (Kajikawa et al., 1990; Larsen et al., 1982; Shivonen et al., 1981) is very similar to other viral infections, but these mechanisms do not eliminate virus from the host. When saying MVV does not affect proliferative responses in sheep most researchers have looked at the lymphoproliferative response of PBMC from MVV-infected sheep to mitogens or MVV antigens *in vitro* (Griffin et al., 1978; Larsen et al., 1982a). A decreased primary immune response to ovalbumin (measured by immunoglobulin production in serum) or DTH response to PPD has been found in MVV-infected sheep *in vivo* (Dr. P. Bird, personal communication), but this was not reflected in the lymphoproliferative response *in vitro*. Therefore there may be an immune response defect *in vivo* and it may occur at the monocyte level. In addition, bacterial

bronchopneumonia in maedi is a common feature in naturally infected sheep with maedi (Markson et al., 1983; Cultlip et al., 1979), which suggests that partial immunosuppression may occur in some MVV-infected sheep. This is at odds with studies on immunity of the respiratory tract in MVV infection which have shown that alveolar lymphocytes, both CD4 and CD8 positive populations are activated (increased MHC class II or decreased CD5) (Cordier et al., 1992; Watt et al., 1992b). Spontaneous release of fibronectin and neutrophil chemotactic factor and MHC class II expression of AM from maedi lungs are also increased in MVV-infected sheep (Cordier et al., 1990) which suggests that AM in MVV infection are more activated than in control sheep.

The reason for the discrepancy in results in MVV infection is not clear. *In vivo*, only a very small proportion of monocytes or macrophages are infected and express viral antigens (Gendelman et al., 1985). Therefore the effect on effector and antigen presenting functions of monocyte/macrophages due to direct viral infection may be very limited. *In vitro*, MVV undergoes productive infection in cultured MDM and so the effect of virus infection in the majority of macrophages and so on total antigen presentation may be studied. In this chapter the antigen presenting function of MVV-infected macrophages, *in vitro*, was evaluated by using antigen specific-T cell lines. The results showed a defect in antigen presenting function of MVV-infected MDM late in virus infection but not early after infection (before day 3 post infection).

6.2 Results

6.2.1 Generation of antigen specific-T cell lines

Two sheep (1203T & 1275T) were used to generate ovalbumin-specific T cell lines. In these experiments, two T cell lines (1203T1 and T4) were generated from sheep 1203T, which were specific for ovalbumin, but not for an irrelevant antigen (PPD). In contrast, the generation of ovalbumin specific T cell lines from

sheep 1275T failed in 3 separate experiments. After 2-3 cycles of antigen stimulation, phenotypic analysis of the T cells from sheep 1275T showed greatly decreased numbers of CD4 lymphocytes and these T cells were not sensitive to ovalbumin. However, a T-cell line (1275T-T4) generated after 2 cycles of antigen stimulation was used in later experiments. The lymphoproliferative response of PBMC and ovalbumin-specific T-cell lines (1203T-T1 and 1275T-T4) are shown in figure 6.1 A & B, respectively.

Ovalbumin-specific T-cell lines were generated from sheep primed with antigen in complete Freund's adjuvant (CFA) which contains heat inactivated *Mycobacterium tuberculosis*. Therefore, the PBMC from these primed sheep not only responded to ovalbumin, but also to purified protein derivative (PPD) (data not shown). But after 2 cycles of ovalbumin and IL-2 stimulation, these cell lines became specific for the original stimulating antigen (ovalbumin), not to antigens in the adjuvant (figure 6.1.B).

PBMC from sheep 1275T which had been primed with ovalbumin in CFA and sheep 1216V which had been primed with BCG showed proliferative responses to PPD. Both sheep were boosted with PPD (50µg) in PBS and the PBMC used to generate PPD-specific T-cell lines. After 2 cycles of antigen then rhIL-2 stimulation, two PPD specific T cell lines were generated from both sheep. However, both PPD-specific T-cell lines showed high backgrounds after 7 days with IL-2 (figure 6.2.B). The background may have been due to IL-2 receptors of the lymphocytes not being completely down regulated after 7 days with rhIL-2. To reduce this background, both lines were submitted to 7 days with antigen and then the IL-2 cycle was prolonged to 10-12 days. The lines were still specific for PPD (heat shock protein 65KD of *Mycobacterium paratuberculosis*, ovalbumin and MVV antigen did not induce responses) and the background was decreased (figure 6.2.C).

Figure 6.1 Characteristics of ovalbumin-specific T cell lines

A. Proliferative response of PBMC to ovalbumin

PBMC prepared from antigen-primed sheep (1203T & 1275T) were cultured with various dilutions of ovalbumin at 1×10^5 cells per well in 96-well flat-bottom microtiter plates for five days at 37°C , 5% CO_2 . Lymphocyte proliferation was measured by ^3H -thymidine incorporation ($1\mu\text{Ci}$ per well) over the last five hours as described in section 2.9.3. Data are expressed as $\text{CPM} \pm$ standard deviation.

B. Proliferative response of ovalbumin-specific T cell lines

Ovalbumin-specific T-cell lines were generated after two (1275T-T4) or four (1203T-T1) cycles of antigen stimulation and IL-2 expansion as described in section 2.9.2. Antigen specific T-cells were cultured at 5×10^4 cells/well with irradiated autologous PBMC (2×10^5 /well) in the presence of various dilutions of ovalbumin or irrelevant antigen (PPD) as indicated at a total volume of $200\mu\text{l}$ at 37°C , 5% CO_2 for 5 days. Proliferation was measured as above.

C. Antigen presenting function of MDM

Autologous MDM (sheep 1203T) were cultured for 12 days in 25cm^2 flasks. MDM were harvested and plated, 1×10^4 cells per well, into 96-well flat-bottom microtiter plates. For comparison, aliquotes of MDM were treated with 50% Con-A supernatant (v/v) for two days before the proliferation assay to increase MHC class II expression. 3×10^4 ovalbumin-specific T-cells (1203T-T1) and various dilutions of ovalbumin were added in a total volume of $200\mu\text{l}$ per well. Cells were cultured for 5 days and the proliferation was measured as above. The T-cell line with irradiated PBMC (2×10^5 cell/well) and antigen as described in B was set up as a positive control. MDM or T cells alone, T cells with antigen (all counts under 1,000 CPM) and irradiated PBMC or MDM with T-cells without antigen (counts were about 2,000 and 5,000, respectively) were set up as background controls. The antigen presenting cells used are indicated.

FIGURE 6.1

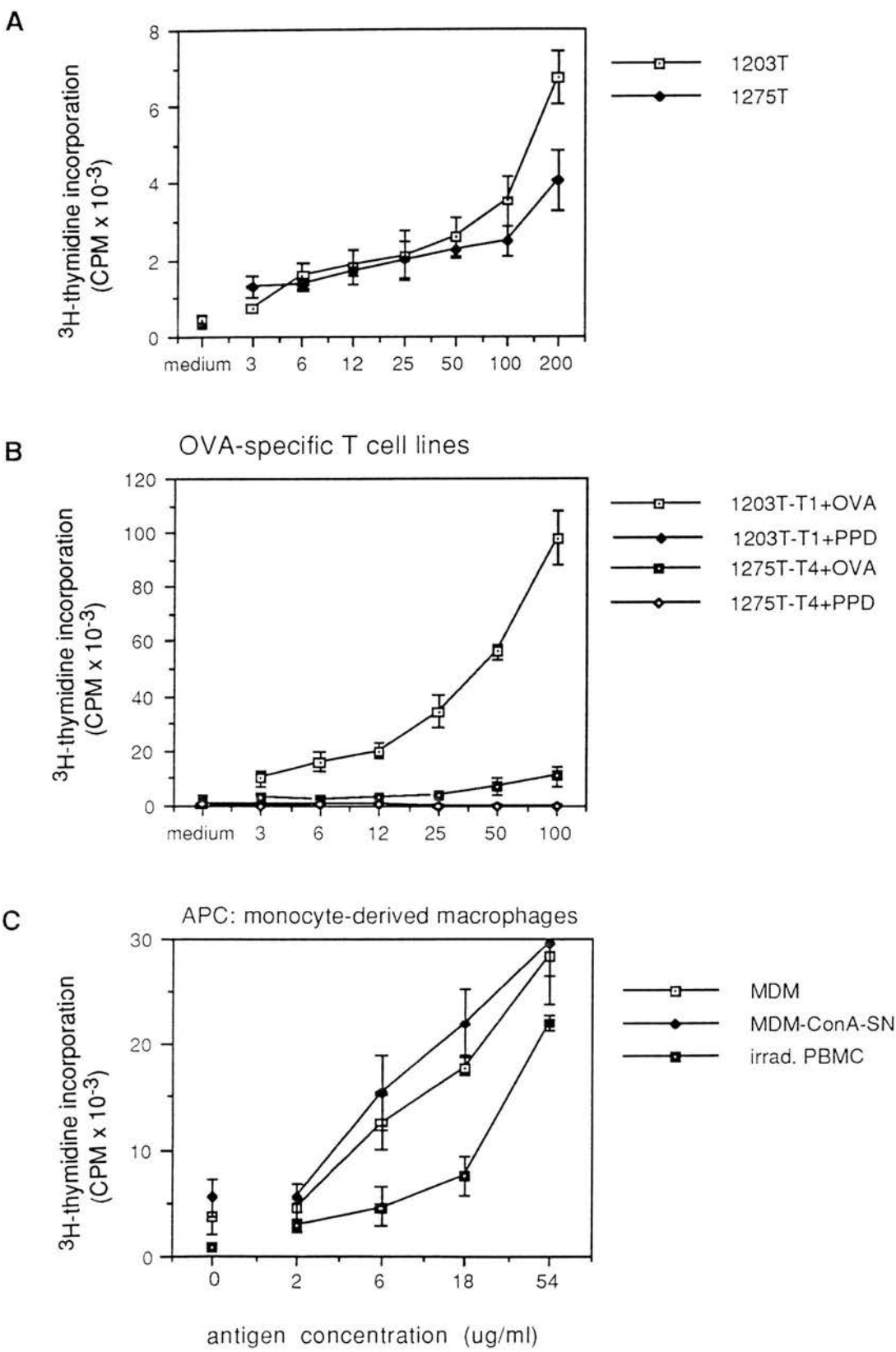


Figure 6.2 Characteristics of PPD-specific T-cell lines

A. Lymphoproliferative response of PBMC from sheep 1216V and 1275T after boosting with PPD. PBMC were cultured 1×10^5 cells per well with serial dilutions of PPD in 96-well flat-bottom microtiter plates for five days at 37°C , 5% CO_2 . Lymphocyte proliferation was measured by ^3H -thymidine incorporation ($1\mu\text{Ci}$ per well) over the last five hours as described in section 2.9.3.

Data are expressed as $\text{CPM} \pm$ standard deviation.

B. PPD-specific T cell lines were generated from sheep 1216V and 1275T after 2 cycles of antigen stimulation and IL-2 expansion (7 days). 3×10^4 PPD-specific T cells, 2×10^5 irradiated autologous PBMC and various dilutions of antigen, either PPD or irrelevant (ovalbumin), in a total volume of $200\mu\text{l}$ were plated into 96-well flat-bottom microtiter plate and then cultured at 37°C , 5% CO_2 for 5 days. Lymphoproliferation was measured as described in section 2.9.3.

Data are expressed as $\text{CPM} \pm$ standard deviation.

C. Antigen presenting function of MDM using PPD-specific T cell lines

The PPD-specific T cell line (1216V-P3) was generated as described in profile B, except the line was expanded in IL-2 for 12 days during the second cycle. 1×10^4 autologous MDM (day 12), 3×10^4 PPD-specific T cells and various dilutions of antigens (as indicated) in a total volume of $200\mu\text{l}$ were plated into 96-well flat-bottom plates and cultured for 5 days at 37°C , 5% CO_2 . Lymphoproliferation was measured as above. T cells with antigen or T cells with MDM in the absence of antigen gave counts under 1,000 CPM.

FIGURE 6.2

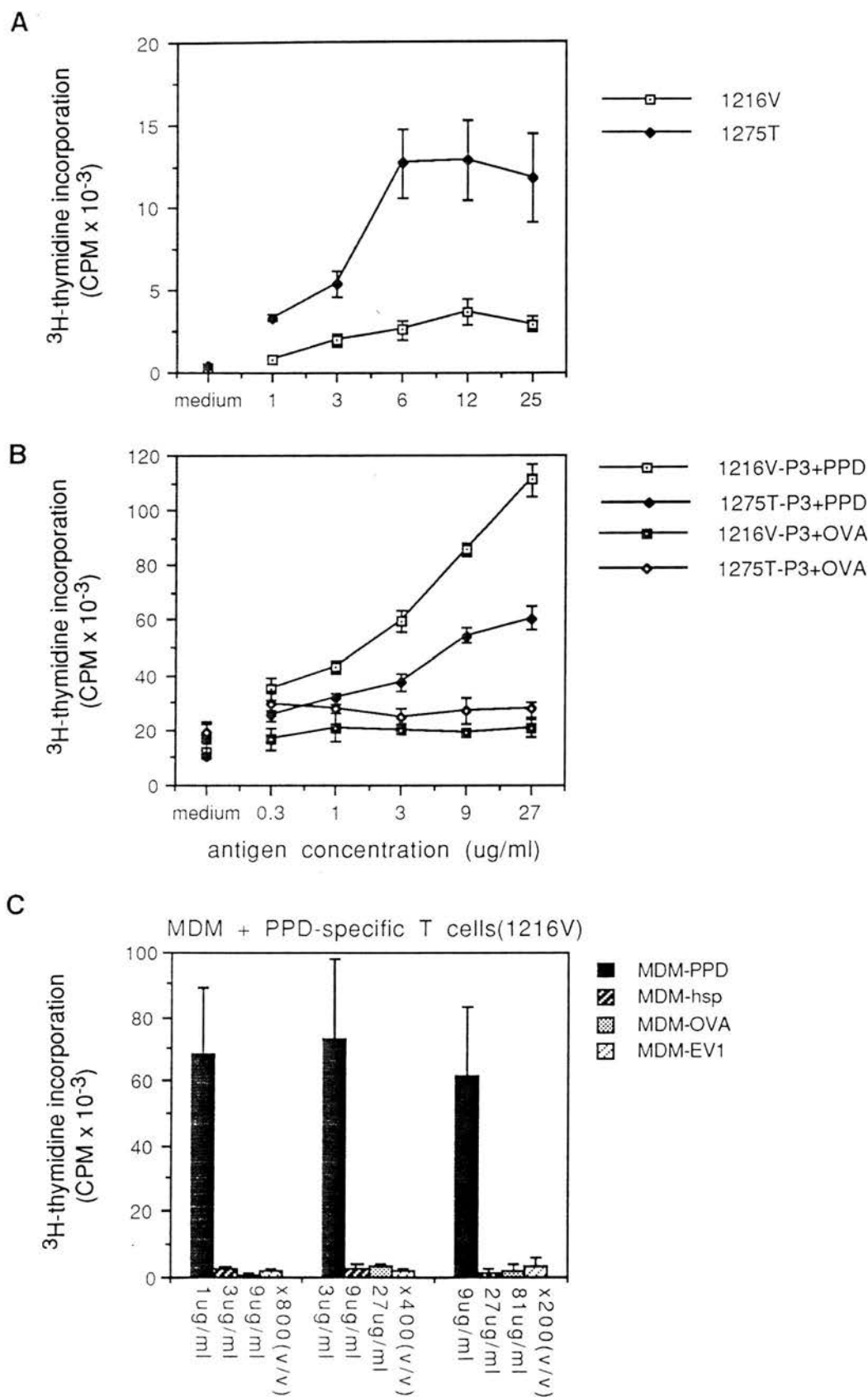


TABLE 6.1 Phenotype of antigen-specific T cell lines

| T cell line* | cycle | antigen specificity | positive cells (%)** | | | |
|--------------|-------|------------------------|----------------------|------|------|-----|
| | | | CD4 | CD8 | Ig | T19 |
| 1203T-T1 | 4 | OVA | 53.8 | 12.6 | 13.0 | 9.6 |
| 1275T-T4 | 2 | OVA | 32.1 | 45.2 | 13.1 | 2.7 |
| 1216V-P3 | 2 | PPD | 66.4 | 11.1 | 1.5 | 8.7 |
| 1275T-P3 | 2 | PPD | 74.5 | 11.8 | 1.4 | 2.0 |

* Antigen specific T cell lines were generated after antigen stimulation and IL-2 expansion of PBMC. The T cell phenotype were analyzed by flow cytometry after IL-2 expansion.

** Cells stained with SBU-T4 (anti-CD4), SBU-T8 (anti-CD8), VPM8 (anti-Ig L-chain) and ST197 (anti-T19).

The phenotype of these antigen-specific T-cell lines is shown in table 6.1. The table shows that the majority of antigen specific cells had expanded the CD4⁺ population.

6.2.2 Antigen presenting function of cultured MDM

Cultured MDM express very low levels of MHC class II molecules on the cell surface (section 4.2.1) and they were next studied to see if they could present antigen to trigger a T cell response. The ovalbumin-specific T-cell line (1203T-T1) was used for the responding cells. Con A-supernatant activated MDM (section 4.2.3) were used as MHC class II positive controls. There was no difference in the antigen presenting function of MDM either previously activated with Con A-supernatant or not (figure 6.1.C). A similar result was also found with PPD-specific T cell lines (data not shown). However, another ovalbumin-specific T cell line (1203T-T4) from the same sheep did not respond to ovalbumin presented by cultured MDM but proliferated with antigen and Con A-supernatant activated MDM (figure 6.5). The lymphoproliferative response of T cell lines was related to the number of stimulating cell (dose-effect) (data not shown). In this study a ratio of MDM : responding cell of 1 : 3-5 showed good proliferative responses.

6.2.3 The effect of MVV infection on the antigen presenting function of MDM

In order to study the effect of MVV infection on the antigen presenting function of MDM, the ovalbumin-specific T cell line (1203T-T1) was used as the responding cell with MVV or mock infected MDM (autologous). The results clearly indicated that an impairment in antigen presenting function of MDM occurred by 5 days postinfection compared to uninfected MDM (figure 6.3.A). Using the cell line 1275T-T4 cell line both MDM at 3 days and 5 days showed a reduction in their ability to present antigen (figure 6.3.C). Con A supernatant treatment of MDM for 2 days before the proliferative assay reduced the difference

in antigen presenting ability of 5 days infected MDM and mock infected MDM (figure 6.3.B). Con A-supernatant may interfere with viral replication in the cell and so these cells may not have reached the same stage in infection as unactivated MDM.

To reduce backgrounds the PPD-specific T cells underwent an IL-2 expansion cycle which was lengthened to 10 days (1216V-P5 and 1275T-P5) before being used as responding cells for MVV-infected MDM. The lymphocytes showed a similar reduction in proliferation to MVV-infected MDM as ovalbumin-specific T cells, one line showed a decreased response to both 3 and 5 day infected MDM (figure 6.4.A) whilst one showed a decrease to only 5 days infected MDM (figure 6.4.B). The defects in presentation of PPD were seen at low antigen concentrations (0.3µg/ml), but were overcome by higher concentrations of PPD (9µg/ml) (figure 6.4).

To address whether the decrease in the antigen presenting capacity of MVV-infected MDM was related to MHC class II expression on the cell surface, the phenotype of MVV or mock-infected MDM after Con A-supernatant activation was investigated. The results show a decrease in MHC class II expression on MVV-infected MDM as early as 3 days post infection compared to ConA-supernatant activated uninfected MDM (table 6.2). Table 6.2 also shows that MDM not treated with ConA-supernatant had little MHC class II expression. This is probably switched on during the 5 days assay with lymphocytes. Not all the lymphocytes are in resting phase when added to the cultures (backgrounds can be high) and so are probably secreting lymphokines which can upregulate MHC class II expression on the surface of MDM.

6.2.4 Antigen presenting function of alveolar macrophages

Due to arthritis, sheep 1203T was put down and so it was possible to obtain AM from BAL. The ability of these fresh AM to present ovalbumin to a

Figure 6.3 Comparison of the antigen presenting function of MDM either infected or mock-infected with MVV

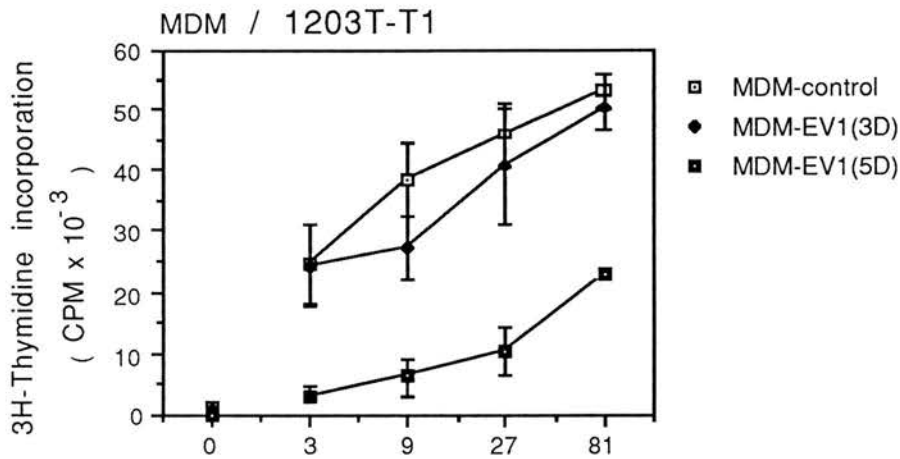
MDM (autologous) were cultured in 25cm² flasks and then infected with MVV at 1 TCID₅₀ per cell on day 7 (—□—) and 9 (—◆—) (as indicated). On day 12 cells were harvested and 1x10⁴ viable MDM were plated into 96-well microtiter plates. 3x10⁴ ovalbumin-specific T cells (1203T-T1 for profile A and B; 1275T-T4 for profile C) and various dilutions of ovalbumin in a total volume of 200μl were added and then cultured for 5 days. Lymphocyte proliferation was measured by the incorporation of ³H-thymidine (1μCi per well) over the last five hours as previously described (section 2.9.3).

Data are expressed as CPM ± standard deviation.

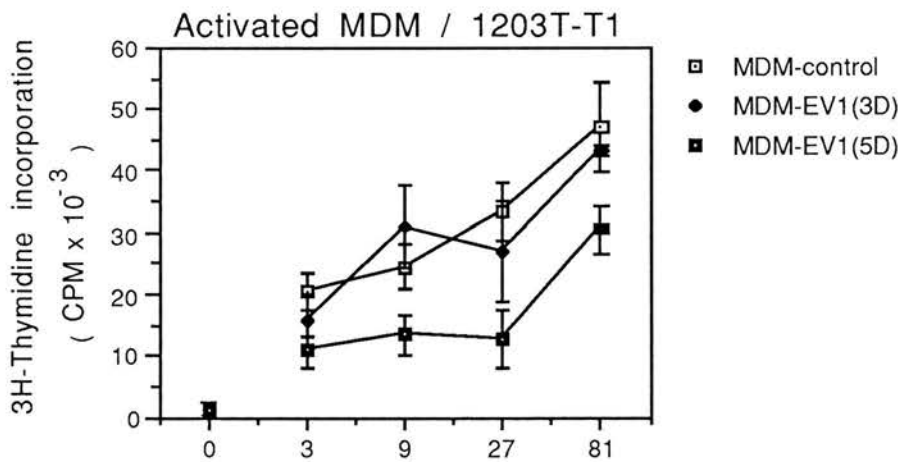
- A. Proliferation of ovalbumin-specific T cells (1203T-T1) with mock or MVV infected MDM without Con A-supernatant treatment.
- B. As A except the cultured MDM had been pre-treated with 50% Con A-supernatant (v/v) for 2 days before the proliferation assay.
- C. Response of ovalbumin-specific T cells (1275T-T4) to mock or MVV infected MDM without ConA-supernatant treatment..

FIGURE 6.3

A



B



C

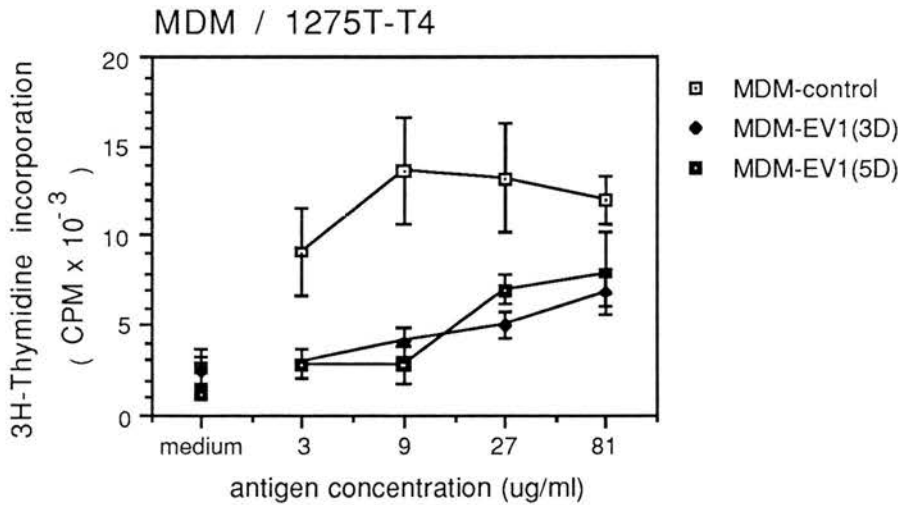


Figure 6.4 Comparison of the antigen presenting function of MVV infected MDM using PPD-specific cell lines

MDM (autologous) were cultured in 25cm² flasks and then infected with MVV at 1 TCID₅₀ per cell on day 7 (—□—) and 9 (—◆—) (as indicated). On day 12 cells were harvested and 1x10⁴ viable MDM were plated into 96-well microtiter plates. 3x10⁴ PPD-specific T cells (1216V-P5 and 1275T-P5 which had gone through 3 cycles of antigen stimulation with a 10 day IL-2 expansion cycle) and serial diluted antigen in a total volume of 200μl were added and then cultured for 5 days. Lymphocyte proliferation was measured by the incorporation of ³H-thymidine (1μCi per well) over the last five hours and measured as previously described (section 2.9.3). Data are expressed as CPM ± standard deviation.

- A. Response of PPD-specific T cells (1216V-P5) to mock or MVV infected MDM.
- B. Response of PPD-specific T cells (1275T-P5) to mock or MVV infected MDM.

FIGURE 6.4

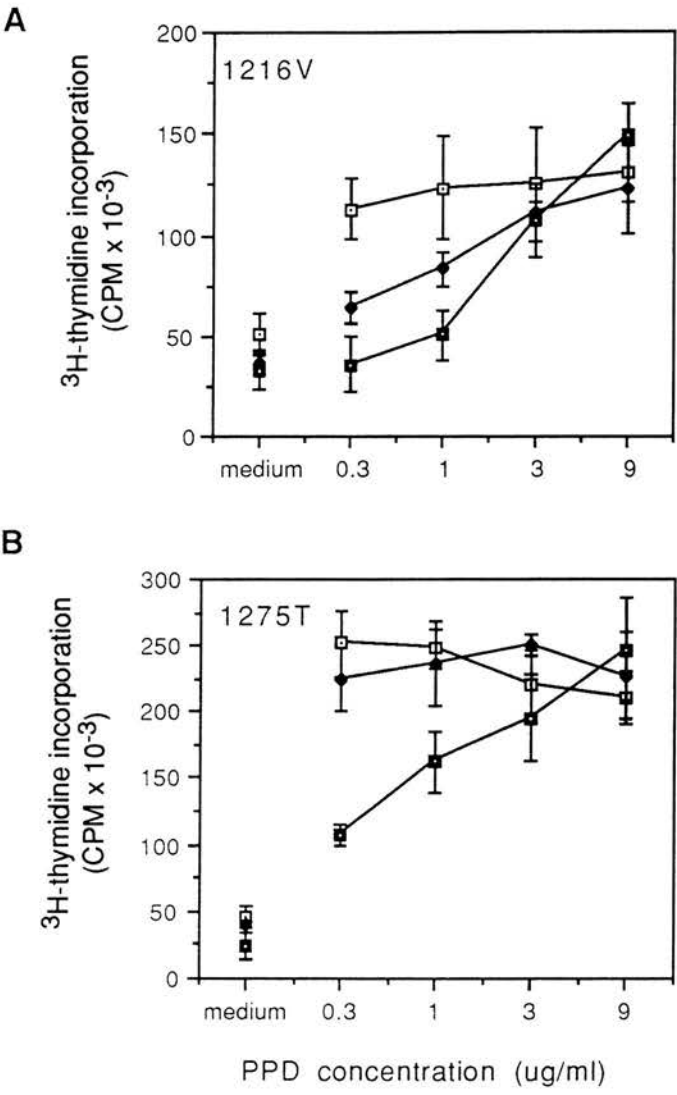


Figure 6.5 Antigen presenting function of alveolar macrophages

2×10^4 AM (sheep 1203T), 3×10^4 cells from the ovalbumin-specific T-cell line (1203T-T4) and various dilutions of ovalbumin were plated into 96-well flat-bottom microtiter plates in a total volume of 200 μ l in each well. Controls included MDM (2×10^4 per well) either Con A-supernatant activated or not activated before the proliferation assay. Proliferation was assayed as previously described in section 2.9.3.

FIGURE 6.5

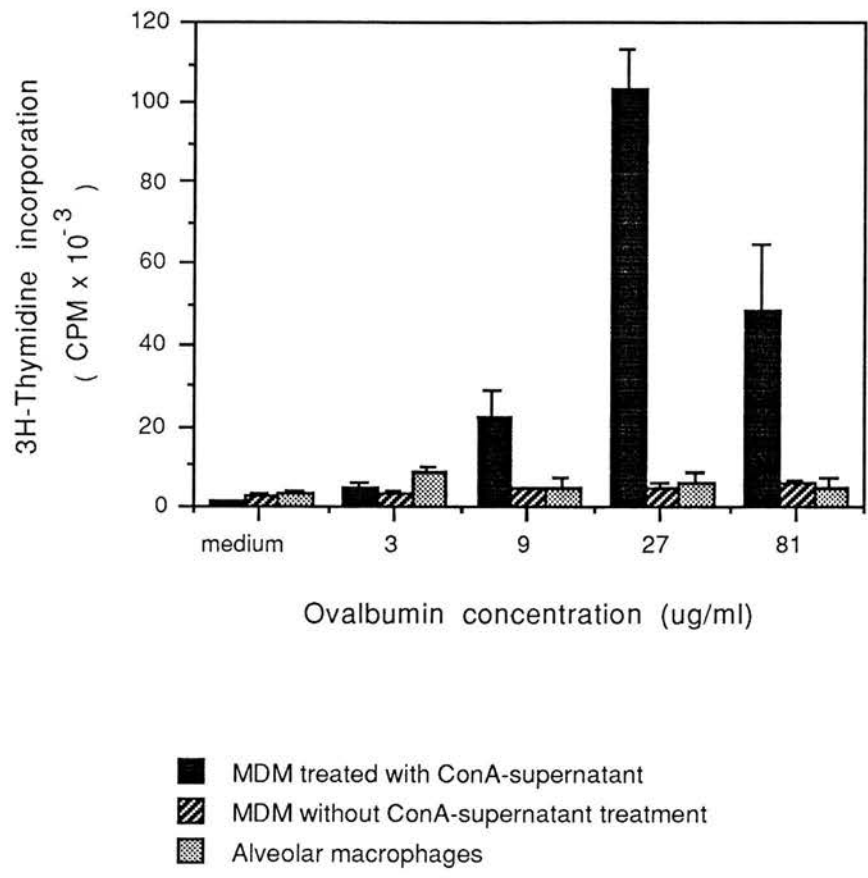


TABLE 6.2 Phenotype of MVV-Infected MDM Treated with Con A-Supernatant*

| Phenotype | Days Post infection** | | | | |
|-----------------|-----------------------|------|------|------|------|
| | Control# | 0 | 3 | 5 | 7 |
| MHC class II DQ | 5.1 | 30.4 | 12.4 | 5.7 | 5.9 |
| MHC class II DR | 4.9 | 32.4 | 12.0 | 9.3 | 9.3 |
| MHC class I | 45.5 | 45.2 | 34.8 | 30.8 | 27.9 |
| CD8 | 17.5 | 2.7 | 10.2 | 9.0 | 10.2 |
| LFA-1 | 31.0 | 38.1 | - | 21.7 | 20.5 |

* MDM were infected with MVV. 1 TCID₅₀ per cell, on day 5, 7, 9 and 11 and their phenotype was analyzed on day 12. 50% Con A-supernatant (V/V) was added to cultures 2 days before analysis.

** Data are expressed as mean fluorescence intensity where negative control fluorescence has been subtracted.

Control MDM were not treated with Con A-supernatant and were uninfected.

1203T cell line was tested in comparison to autologous cultured MDM. The results show that AM could not efficiently stimulate lymphoproliferation of an ovalbumin-specific T cell line (1203T-T4) compared to activated MDM (figure 6.5). The AM used in this assay expressed moderate levels of MHC class II DQ (MF=41.2) and DR (MF=50.3) on their cell surface as detected by flow cytometry (data not shown).

6.4 Discussion

The purpose of this study was to evaluate the antigen presenting capacity of macrophages for inert antigen after infection with MVV. Factors affecting the lymphoproliferative response are at three different levels: antigen uptake and processing, antigen presentation with MHC class II and accessory factors (cytokines) released by macrophages. PBMC and partially purified lymphocytes from PBMC may contain dendritic cells, monocytes and B cells which can all act as antigen presenting cells. Therefore to study the antigen presenting ability of macrophages alone, generation of antigen-specific T cell lines was used.

It has been reported that activated sheep lymphoblasts respond well to recombinant human IL-2 (rhIL-2) (Bujdoso et al., 1989). T-cell lines can be generated by antigen stimulation followed by rhIL-2 expansion for 2 weeks by which time activated cells have gone back to a resting stage (Bujdoso et al., 1989). However, in my hands ovalbumin-activated T-cells could not survive in culture with rhIL-2 for two weeks, especially lymphocytes from sheep 1275T. Most activated T-cells underwent "programmed cell death" (PCD) (Cohen, 1991) after 2 weeks in culture with rhIL-2. In contrast, with PPD specific T-cell lines some cells survived in culture in the presence of rhIL-2 for 10-12 days. However the majority of T cells eventually underwent apoptosis which led to a loss of cells during late phase of this cycle. Culturing PPD-specific blasts with IL-2 for 7 days may not allow enough time for all IL-2 receptors to be down regulated and this may be the cause of high backgrounds in proliferative assays with PPD cell lines. To reduce backgrounds a

balance between the length of culture with IL-2 and surviving cells was determined by microscopic observation.

Two ovalbumin-specific T cell lines were generated from sheep 1203T, but not from sheep 1275T. The reason for this difference is unclear but may be due to immune response genes in different sheep. Sheep 1203T PBMC did respond better to ovalbumin than 1275T. It is unlikely to be the absence of costimulating molecules in cultures (Weave and Unanue, 1990) as PPD lines could be made from sheep 1275T. Poor presentation of ovalbumin on the MHC class II molecules of 1275T is probably the cause. This is supported by the fact that the only ovalbumin specific T cell line to be generated from this sheep was composed mainly of CD8⁺ lymphocytes and had lost most of its CD4⁺ lymphocytes (Table 6.1).

Most antigen presentation on MHC class II is thought to occur on newly synthesized class II molecules (Neefjes et al., 1990). Therefore both unactivated and Con A supernatant activated MDM need to synthesize MHC class II to stimulate T cell proliferation. With activated MDM this is likely to occur as the cells are expressing high levels of class II when they are added to the lymphocytes. Unactivated MDM express little detectable class II when they are added to lymphocytes and so MHC class II expression must be switched on by culture with the lymphocytes but before the T cells have interacted with large amounts of presented antigen. The T cell line in this case may spontaneously release cytokines, such as IL-2 and IFN- γ into the cultures (Paulnick, 1992; Katz and Michalek, 1991; Torres et al., 1982) which would upregulate expression of MHC class II on the macrophages (Sztein et al., 1984), allowing presentation of the antigen which was present continuously. One T cell line (1203T-T4) could not use unactivated MDM as antigen presenting cells. The most probable explanations for this are that this line was truly resting and therefore not producing sufficient lymphokines to allow MHC class II expression on macrophages or that this T cell line produced lymphokines incapable of upregulating MHC class II on macrophages. Activated Th1 cells

produce IL-2 and IFN- γ , whilst Th2 cells produce IL-4 (Eastcott et al., 1990; Finkelman et al., 1990). Th2 cell responses are strictly dependent on accessory cell expression of IL-1, compared to Th1 proliferation (Weaver et al., 1988). The lymphokine profiles of the T cell lines used here have not been investigated but this would be of interest to these results.

The antigen presenting function of MVV-infected MDM was found to decrease by 3 to 5 days postinfection when ovalbumin or PPD T cell lines were used as responding cells. This result paralleled those from the phagocytic assays (chapter 5). There may therefore be a fault in antigen uptake and processing in productively infected macrophages. There was also reduced expression of MHC class II even after Con A supernatant activation in MVV-infected MDM (table 6.2) which could contribute to the decreased antigen presentation. Whether there is also a decrease in costimulatory factors released by MVV-infected MDM, compared to uninfected MDM, in early or productive infection is unknown.

Ennen et al (1990) reported a decrease in IL-1 production from HIV-infected MDM in response to anti-CD3 monoclonal antibody stimulation compared to uninfected MDM, but this is not seen on LPS stimulation. However, the decrease in IL-1 secretion by HIV-infected cells *in vitro* occurs after 3 weeks post infection which is longer than the infection times used here for MVV infected MDM. In addition, the decrease in antigen presenting function of MVV-infected MDM seen here *in vitro* may not parallel the situation *in vivo* where lymphoproliferation is a hallmark of MVV induced lesions (Cutlip et al., 1979; Olive et al., 1981b).

It has been reported that there is a decrease in MHC class II expression and accessory cell function in HIV-infected MDM or macrophage hybridomas which is related to reverse transcriptase activity and p24 levels (day 21-24 post infection) in the culture (Petit et al., 1988; Ennen et al., 1990; Sperber et al., 1993). A defect in accessory cell function of monocytes in stimulating lymphocyte proliferation for recall antigens (Rich et al., 1988), anti-CD3 monoclonal antibody (Melendez-

Guerrero et al., 1991), or in mixed leukocyte reactions (MLR) (Twigg III et al., 1991) has been reported in HIV infected patients, especially those patients with ARC or AIDS. The defect in MLR reactions stimulated by monocytes is paralleled to the decrease in MHC class II expression on these cells (Heagy et al., 1986; Sei et al., 1986). This is similar to the results seen here with MVV. Lacroix et al (1993) reported that a defect in accessory cell function in HIV-infected MDM occurred as early as 2 days post infection. However, this defect is limited to low MDM : T cell ratios using anti-CD3 monoclonal antibody stimulation, but is not seen with other mitogens or recall antigens (Ennen et al., 1990). The MDM : T cell ratio used in the above study may not be physiological. Here, MVV replication is lytic in MDM which is not the same as the chronic HIV replication seen in MDM or the U937 cell line.

Part of the pathogenesis of HIV disease includes a decreasing memory T cell population which might have resulted from decreased monocyte accessory function when recall antigens were used (Rich et al., 1988). Results from cultures with HIV infected PBL and monocytes are complicated by the fact that lymphocytes may be infected with HIV at the start or become infected with HIV from virus-infected monocytes during the culture. Lymphocytes may therefore not respond to antigen stimulation because of HIV infection or interference by gp120 present in the culture (Mann et al., 1990b). This phenomenon may be more evident *in vitro* when MDM or U937 cells are highly infected and produce more viral particles which may infect or interfere with lymphocytes. Therefore, the decrease in accessory cell function in HIV infection may not only be due to monocytes, but also to the infection or interference of lymphocytes. This is supported by a mump-specific T cell line which did not indicate an impairment in accessory cell function of monocytes from HIV-infected patients (Twigg III et al., 1991).

During MVV disease progression there is enhancement of MHC class II expression on macrophages (Lujan et al., 1993; Harkiss et al., 1991; Cordier et al.,

1990). This suggests that there may be an increase in accessory cell function of AM in antigen presentation. However, AM taken from sheep 1203T did not efficiently stimulate T-cell proliferation when compared to activated MDM. Suppression of T-cell proliferation caused by AM has been reported (Holt et al., 1985 & 1986; Fu and Blankenhorn, 1992), but this effect may vary in different species (Holt et al., 1986). The suppressive effect of AM is mediated by the nitric oxide synthase pathway (Holt et al., 1993; Mills, 1991; Fu and Blankenhorn, 1992) and this pathway can be amplified by treatment of AM with IFN- γ , TNF- α , IL-1, and endotoxin (Ding et al., 1988; Nussler et al., 1992; Stuehr and Marletta, 1987). Therefore, the increased activation state of AM in MVV-infections (Cordier et al., 1992; Lujan et al., 1993) suggests not only an enhancement of accessory cell functions of AM but also an increase in the release of more nitric oxide in MVV infected sheep which may in turn inhibit local pulmonary immunity. However accessory cell functions and suppressive effects of AM in sheep have not been well studied. Indeed in HIV and tuberculosis there is increased HLA-DR expression and IL-1 release by AM (Ina et al., 1991; Buhl et al., 1993; Soliman et al., 1992) but infections of the lung are not cleared and so measures of AM activation are not necessarily linked to increases in effective pulmonary immunity.

6.4 Summary

Antigen specific T-cell lines were generated from antigen primed sheep after 2 cycles of antigen then IL-2 stimulation. The period of IL-2 stimulation was varied for different cell lines. Cultured MDM could efficiently present antigens to antigen specific T cell lines. The interaction of T-cell lines and MDM played a role in the activation of MDM function. Productively MVV-infected MDM showed a decrease in their antigen presenting capacity (3-5 days postinfection). AM did not present antigen well to a T cell line.

CHAPTER 7

CELL-MEDIATED CYTOTOXICITY IN MAEDI VISNA VIRUS INFECTION

7.1 Introduction

After viral infection, the host develops a humoral and cell-mediated immune response. The development of specific antibodies or cytotoxic T lymphocytes (CTL) is specifically programmed to eliminate free virus and virus-infected cells (Sissons and Oldstone, 1980; Kohl, 1991). Natural killer (NK) cells, monocytes/macrophages and neutrophils can directly lyse virus infected cells or tumor cells in the absence of specific antiserum (Graziano and Fanger, 1987; Yokoyama, 1993). Both virus-specific and non-specific defense mechanisms have important roles against viral infection. Usually, the development of virus-specific cytotoxic activity mediates the clearance and so resolution of virus infection. However in some cases virus specific cytotoxicity may mediate pathological lesions as in the case of adult mice infected with lymphocytic choriomeningitis virus (Leist et al., 1988). Lentivirus infections are unusual (Narayan and Clements, 1989) in that the virus can persist in target cells, even though the host has developed virus-specific CTL and antibody.

High frequencies of HIV-specific CTLs have been found in HIV patients, especially at the asymptomatic stage (Walker et al., 1987 & 1988; Riviere et al., 1989; Nixon et al., 1988; Vanham et al., 1990; Hoffenbach et al., 1989), but in late phases of HIV infection, especially in the patients with AIDS, CTL activity against HIV is markedly decreased (Vanham et al., 1990; Carmichael et al., 1993; Clerici et al., 1991). Class I restricted CTL activity directed against HIV *env*, *pol*, *gag*, *nef*, *rev* and *vif* products has been seen (Koup et al., 1989; Nixon et al., 1988; Michel et al., 1992; Lysterly et al., 1987; Walker et al., 1987; Riviere et al., 1989; Buseyne et al., 1993; Clerici et al., 1991). Virus-specific, MHC class II restricted, CD4⁺ T cell mediated cytotoxic activity has also been documented in HIV infections (Orentas et al., 1990; Littaua et al., 1992; Curiel et al., 1993) or antigen primed mice (Michel et al., 1992). Virus-specific CTL can be detected in blood (Hoffenbach et al., 1989; Koup et al., 1989), bronchoalveolar lavage (BAL) cells (Plata et al., 1989; Autran et

al., 1991) and CSF (Sethi et al., 1988) in HIV-infected patients suggesting good cellular immunosurveillance within the infected host. However, HIV genetic variants which can escape cytotoxic T cell recognition have been reported in a longitudinal study (Phillips et al., 1991). The mutation of virus therefore, provides a pathway for escaping immunosurveillance. With SIV, similar results have been shown with high frequencies of CTL for the *nef* gene product (Bourgault et al., 1992).

Non MHC-restricted CD16⁺ lymphocyte mediated cytotoxicity has been described (Riviere et al., 1989; Weinhold et al., 1988) that is able to kill gp120 coated or HIV-infected target cells. Patients with AIDS have been shown to have defective NK activity (Poli et al., 1985; Rook et al., 1985; Scott-Algara et al., 1992), but this defect can be modified by adding IL-2 to cell cultures (Rook et al., 1983 & 1985). These activities are very important in controlling viral replication.

HIV-infected macrophage or monocyte cell lines can be lysed by MHC restricted virus-specific CTL, non-MHC restricted NK cells or ADCC (Plata et al., 1987; Vanham et al., 1990; Autran et al., 1991). The interaction of macrophages and these cytotoxic activities indicates that virus infected macrophages present viral antigen to initiate the immune response and that these cells are eliminated by activated virus-specific or non-specific cytotoxic cells. *In vivo*, HIV persistence in macrophages can be detected at all stages of infection (Ho et al., 1989). Macrophages harboring virus but evading host immunosurveillance are therefore important in the pathogenesis of HIV infection.

Monocyte/macrophages are the predominant infected cells in MVV infection. No decrease in lymphocyte, dendritic or monocyte cell numbers in MVV infected sheep have been reported. Virus specific and MHC-restricted CTL activity can be detected in all MVV infected sheep by stimulating PBMC with MVV-infected skin fibroblasts. (Blacklaws et al., 1994), but direct cytotoxic activity in acute or persistent infections is very difficult to detect (Bird et al., 1993; Blacklaws

et al., 1994). This is different to the direct cytotoxic activity seen in PBMC from HIV infected patients. There is limited viral antigen expressed on infected macrophages *in vivo* (Haase et al., 1976; Gendelman et al., 1985) and so there may not be sufficient antigen to activate circulating precursors in PBMC to show CTL activity *in vitro*. Studies on pulmonary immunity in MVV infection have shown greatly increased numbers of CD8 lymphocytes and decreased CD4/CD8 ratios in BAL fluids of maedi lungs (Lujan et al., 1993; section 4.2.7). Most of these lymphocytes are activated (Cordier et al., 1992; Watt et al., 1992b) which may indicate lymphocytes with either CTL activity or suppressive effects on pulmonary immunity in MVV infection.

It has been shown that skin fibroblasts are good target cells in CTL assays with MVV (Blacklaws et al., 1994). However *in vivo* the important target cell will be the infected macrophage. Therefore the ability of CTL to lyse MVV infected macrophages was studied. In addition, NK or LAK mediated cytotoxic activity is also very important in controlling tumors or virus-infected cells during early viral infection. The role of NK or LAK cells in controlling and destroying MVV infected macrophages was investigated.

7.2 Results

7.2.1 *In vitro* activated MVV-specific, cytotoxic T lymphocytes

Due to line breeding within breeds of sheep, we were not certain that sheep from the same flock would express different haplotypes. Therefore skin cells derived from different sheep were tested their ability to present antigen to PBMC derived CTLs. Results with CTL from 2 different infected sheep are shown in Table 7.1. Many experimental sheep which came from the same flock appeared to share MHC alleles and could present viral antigens to the CTLs. Sheep 1235T showed the lowest cross reaction with both sheep 1071P and 1091P. Sheep 1238T also showed low cross reaction with sheep 1091P.

Using these and similar results to select heterologous sheep from which to isolate MDM, the ability of CTL to recognize and kill MVV infected macrophages was analyzed. After stimulation of PBMC from MVV infected sheep (1071P, 1091P, YT40 and 865A), the majority of lymphocytes were CD8⁺ lymphocytes (ranged between 78% and 62%). These cell populations from all 4 infected sheep contained activated CTL. They all showed virus specific activity for autologous MVV-infected skin fibroblasts, but not heterologous MVV-infected skin fibroblasts (figure 7.1.A, C, E, G). Similar results were also detected using MVV-infected MDM (figure 7.1. B, D, F, H) indicating that the cytotoxic activity is MHC restricted on both fibroblasts and macrophages. However levels of non-specific cytotoxic activity were often high on uninfected macrophages (10-35%), especially autologous macrophages. The levels of non-specific activity varied in different sheep. Sometimes non-specific cytotoxicity was also seen on autologous uninfected skin fibroblasts rather than heterologous skin fibroblasts (figure 7.1.A, C), especially in high effector to target ratios.

In the above assays, autologous MDM were derived from the infected sheep. These macrophages may therefore have expressed low levels of MVV antigen without infection *in vitro* due to MVV persistence in monocytes *in vivo*. Indeed when MDM from MVV infected sheep were stained for *gag* antigen (p15) occasional cells were *gag* positive (less than 1%, data not shown). Therefore some of the background non-specific killing of autologous MDM seen above may in fact be viral specific. To test this hypothesis MDM were derived from an uninfected sheep whose skin cells were known to cross react with the effector lymphocytes (1238T MDM with 1071P CTLs, see table 7.1). The results showed similar levels of nonspecific lysis with uninfected autologous (1071P) and cross reacting (1238T)

TABLE 7.1 Cross-Reactivity of Different Target Cells (Skin Fibroblasts) in CTL Assays

| Target cells (skin fibroblasts) | Specific lysis (%) | | | |
|------------------------------------|---------------------|------|-------------|------|
| | 1071P CTL* | | 1091P CTL | |
| | T : E ratio | | T : E ratio | |
| | 1:25 | 1:50 | 1:8 | 1:25 |
| Mock-infected | | | | |
| 1071P | 2.6 | 6.0 | ND | ND |
| 1091P | ND** | ND | 1.1 | 4.1 |
| 1213T | 9.9 | 11.0 | ND | ND |
| 1220T | 12.0 | 14.0 | -1.2 | 4.5 |
| 1231T | -4.2 | 1.5 | 2.3 | 3.3 |
| 1235T | 2.9 | 5.8 | -3.4 | -1.2 |
| 1238T | 8.0 | 11.1 | -2.4 | -0.1 |
| 1262T | 6.1 | 8.1 | 3.3 | 3.0 |
| MVV-infected | | | | |
| 1071P | 57.1 | 59.6 | ND | ND |
| 1091P | ND | ND | 31.4 | 36.0 |
| 1213T | 56.7 | 69.1 | ND | ND |
| 1220T | 54.3 | 56.4 | 12.4 | 18.0 |
| 1231T | 72.8 | 79.5 | 14.1 | 26.5 |
| 1235T | 3.1 | 4.7 | 1.2 | 2.6 |
| 1238T | 47.1 | 49.5 | 3.9 | 9.0 |
| 1262T | 61.4 | 63.7 | 11.3 | 19.8 |

* PBL (1071P) were stimulated on MVV infected skin fibroblasts in the presence of rh-IL-2 for 2 weeks. 1091P CTL (figure 7.1C) resuscitated from liquid nitrogen were cultured on MVV-infected skin fibroblasts for another one week and then used in CTL assays. Spontaneous release was always less than 31% of the maximal release. Results have been expressed as specific lysis, that is specific ^{51}Cr release.

** ND: not done

Figure 7.1 Detection of MVV-specific, MHC restricted cytotoxicity

PBL from MVV-infected sheep (A&B sheep 1071P; C&D: sheep 1091P; E&F: sheep YT40; G&H: sheep 865A) were cultured on MVV-infected autologous skin fibroblasts (SK) in the presence of rh-IL2 for 2 weeks as described in materials and methods (section 2.10.1). Target cells (1×10^4 per well), autologous (auto) and heterologous (hetero) SK (A, C, E, G) and MDM (B, D, F, H), in 96-well flat-bottom microtiter plates as indicated were infected or mock-infected with MVV, 0.5 TCID₅₀ (SK) or 1 TCID₅₀ (MDM), for 1 (SK) and 2 days (MDM), respectively. Cells were labeled with 2.5 (A-D) or 1.5 (E-H) μ Ci ⁵¹Chromium in the well overnight, washed and used in cytotoxicity assays (16 hours assay) at 3 different target and effector ratios as indicated.

Results have been expressed as % specific ⁵¹Cr release. The percentage spontaneous ⁵¹Cr release from targets compared to maximal release was: A: 19-23%; B: 21-23%; C: 18-22%; D: 28-32%; E: 20-23%; F: 25-31%; G: 18-21% H: 28-31%.

FIGURE 7.1

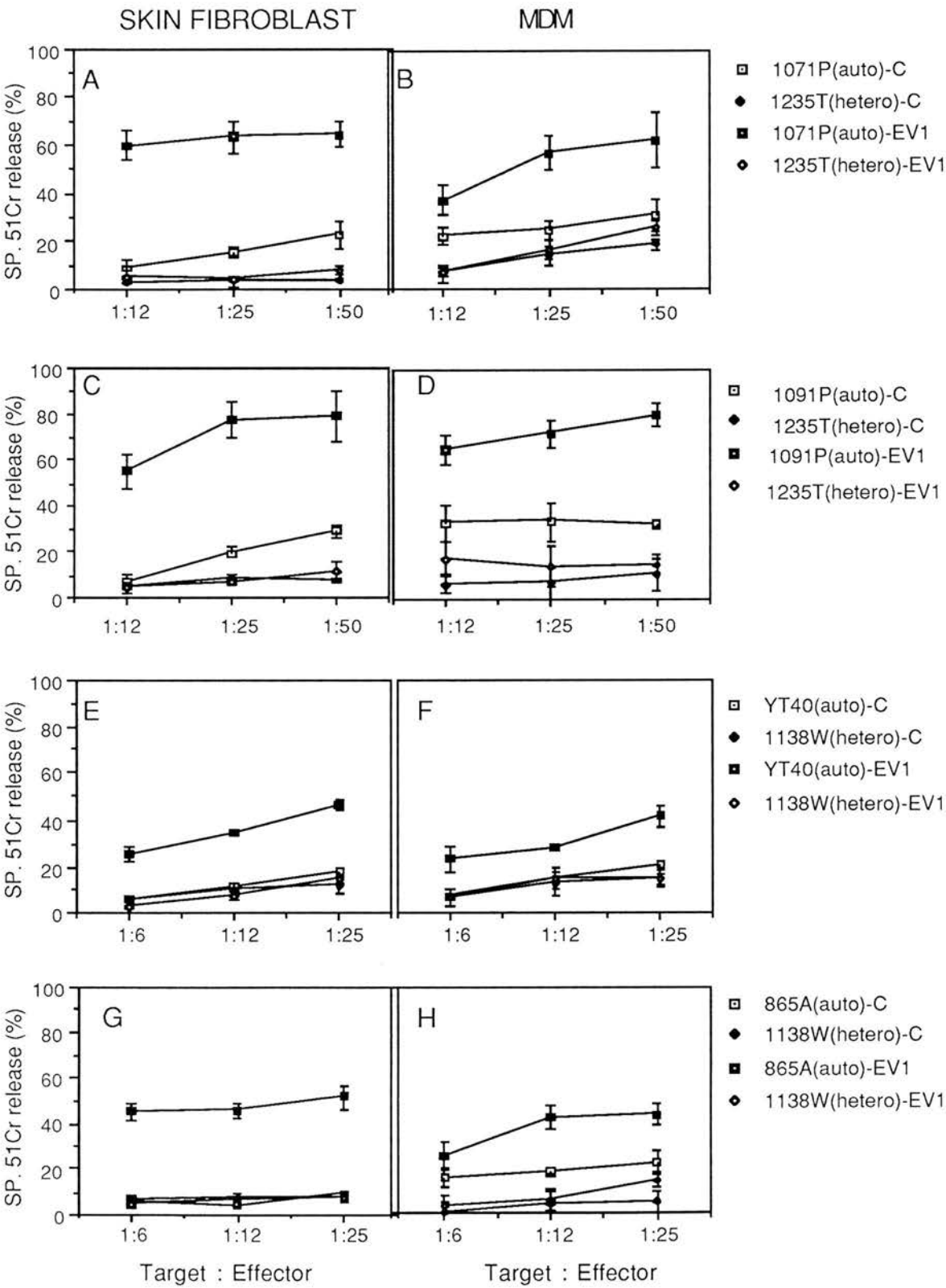
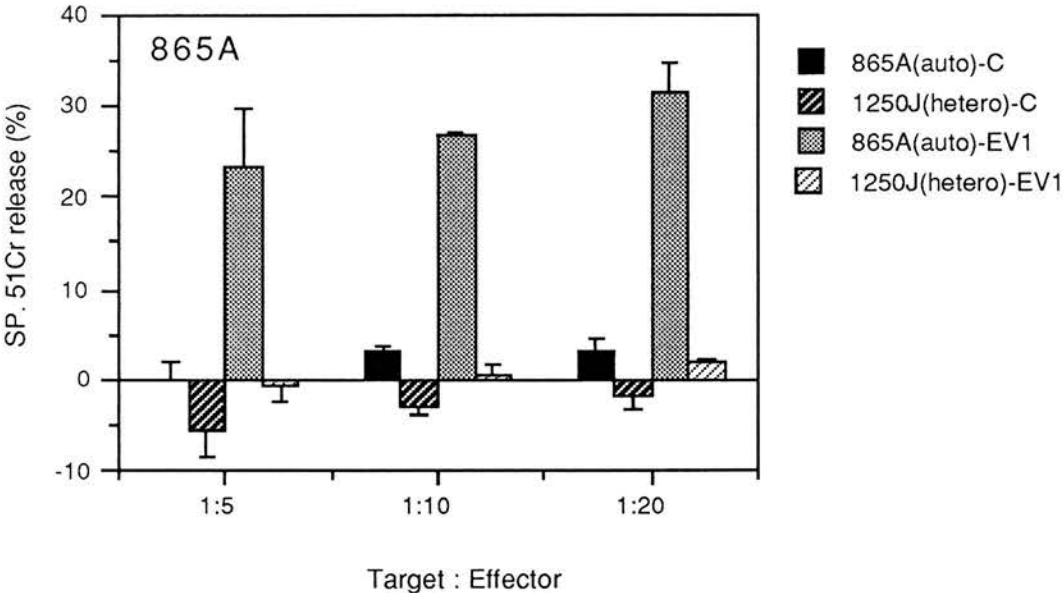


Figure 7.2 Recognition of alveolar macrophage targets

Effector cells (865A PBL) had been stimulated on MVV-infected autologous skin fibroblasts (SK) in the presence of IL-2 for 3 weeks. Autologous (865A) and heterologous (1250J) AM were resuscitated from liquid nitrogen, cultured in 25cm² flasks and then infected with MVV, 1 TCID₅₀ per cell for 2 days. AM were labeled with ⁵¹Chromium, 100μCi per flask, overnight. After four washes, 2x10⁴ AM were plated into 96-well flat-bottom microtiter plates as target cells. Effector cells were added at 3 different target : effector ratios as indicated and the cytotoxic assay was incubated for 6 hours.

Results have been expressed as % specific ⁵¹Cr release. The spontaneous ⁵¹Cr release from target cells compared to maximal release was 21-24%.

FIGURE 7.2



MDM targets (data not shown). Therefore the non-specific cytotoxicity seen in autologous MDM was not caused by virus infection of the cells *in vivo*.

Although recognition of MDM had been shown, *in vivo* the natural target for CTLs, especially in sheep with maedi, might be expected to be AM. Therefore AM were used as targets for *in vitro* stimulated CTLs from blood. MVV-infected autologous AM were specifically lysed by CTL compared with autologous uninfected AM and heterologous AM (figure 7.2). AM spontaneously release was often high and so assay incubations were reduced to 6 hours.

7.2.2 CD8⁺ lymphocytes mediate the CTL activity

To investigate the phenotype of the CTL, CD4⁺ or CD8⁺ lymphocytes were depleted from cultured cell populations by complement. The results (sheep 1071P) are shown in table 7.2. CD4⁺ depleted effectors (CD8⁺=92.6%; CD4⁺= 0.2%) lysed MVV-infected autologous MDM and skin cells. However, there was some nonspecific lysis of autologous uninfected targets. In contrast, the levels of specific lysis on MVV-infected autologous MDM and skin fibroblasts mediated by CD8⁺ depleted CTL (CD8⁺=0.2%; CD4⁺=71.1%) were greatly decreased, suggesting that cytotoxicity was mediated by CD8⁺ CTL. When MDM targets were compared to skin cells targets, depletion of CD8⁺ cells was seen to completely inhibit lysis of MVV infected skin cells. However, there was still greater lysis of MVV infected than mock infected MDM with this depleted population suggesting another cell may also be involved, in a minor fashion, in CTL activity (perhaps CD4⁺ cells). Interestingly, the nonspecific lysis of uninfected autologous targets (both MDM and skin cells) was also dramatically decreased, suggesting that the nonspecific lysis, in part, was mediated by CD8⁺ cells. A similar result was also seen in sheep 865A (data not shown).

TABLE 7.2 CD8⁺ Lymphocyte Mediated Cytotoxicity (Sheep 1071P)

| Targets (autologous) | T : E ratio | specific lysis (%) | | |
|-------------------------|------------------|--------------------------------|-------------------|-------------------|
| | | IgG _{2a} depletion | CD4* depletion | CD8* depletion |
| Mock infected | | | | |
| MDM | 1 : 8 | 13.2 | 17.3 | 12.0 |
| | 1 : 16 | 16.4 | 23.8 | 14.1 |
| MVV infected | | | | |
| MDM | 1 : 8 | 36.2 | 37.0 | 14.9 |
| | 1 : 16 | 44.5 | 47.4 | 24.2 |
| Mock infected | | | | |
| Skin cells | 1 : 8 | 18.0 | 20.6 | 2.0 |
| | 1 : 16 | 24.4 | 21.7 | 4.6 |
| MVV infected | | | | |
| Skin cells | 1 : 8 | 45.6 | 49.1 | 4.2 |
| | 1 : 16 | 48.7 | 54.1 | 5.6 |
| positive | | | | |
| cells (%)** | CD4 ⁺ | 25.9 | 0.2 | 85.5 |
| | CD8 ⁺ | 70.5 | 92.6 | 14.4 |

* PBL were stimulated on MVV-infected skin fibroblasts in the presence of rhIL-2 for 2 weeks as described in materials and methods (section 2.11.1). Activated CTL were incubated with monoclonal antibody, IgG_{2a}, SBU-T4 (anti-CD4), SBU-T8 (anti-CD8), followed by complement depletion before use in assays. The spontaneous release was between 20.2 and 25.7%. Results have been expressed as specific lysis, that is specific ⁵¹Cr release.

** Cell phenotype was analyzed by flow cytometry.

7.2.3 Generation of MVV-specific cytotoxic T lymphocytes using autologous macrophages

To address whether MVV-infected macrophages can stimulate virus-specific precursors *in vitro*, autologous MDM (figure 7.3.A: sheep YT40) and AM (figure 7.3.B: sheep 1257J) infected with MVV were used as stimulating cells to activate CTLs from PBMC. After 2 weeks activation, the CD8⁺ lymphocyte population was increased in both cultures to 65% (YT40) and 66% (1257J) respectively. An apparently MHC restricted CTL activity was detected using autologous, virus-infected skin fibroblasts (YT40, data not shown) or MDM (figure 7.3.A & B). Levels of non-specific lysis of autologous uninfected MDM could be high (around 15-30%) as was also seen with lymphocyte effectors cultured on skin cells.

7.2.4 Cytotoxic activity of alveolar lymphocytes from MVV-infected sheep

Only a very low frequency of MVV infected sheep show direct CTL activity by PBMC (Blacklaws et al., 1994). To investigate whether alveolar lymphocytes can mediate CTL activity without *in vitro* stimulation, 3 sheep (YT40, 865A and 111) which had been infected with MVV more than 4 years were used to obtain bronchoalveolar lavages. None of these sheep showed clinical symptoms and the lungs had no gross lesions. Alveolar lymphocytes were collected and purified by removing adherent cells, twice, onto plastic tissue culture flasks or plates. The AM-depleted alveolar lymphocytes were 85-90% lymphocytes and were then used as effector cells. Fresh AM (data not shown), skin fibroblasts and MDM, either autologous or heterologous, were used as target cells, but there was no virus-specific MHC restricted cytotoxic activity detected with most sheep (figure 7.4.A, B, C). Low levels of CTL activity using skin fibroblast targets were seen in sheep YT40 (figure 7.4.B), but the difference between uninfected and infected targets did not exceed 10%. Using unstimulated PBMC, high effector to target ratio (100:1) are needed to see specific lysis of MVV infected cells. Here these levels were never

reached as lymphocytes are a minor population in lung fluids. The lack of CTL activity here may be for this reason.

Alveolar lymphocytes from the sheep 865A were also stimulated on MVV-infected skin fibroblasts for 12 days in the same manner as previously described and then used as effector cells in a cytotoxic assay. Only a low level of cytotoxic activity was detected on MVV-infected autologous MDM (T:E = 1:10) compared to heterologous targets (figure 7.4.D) which may be due to the low number of effectors recovered after culture. Also non-specific cytotoxicity seen with autologous MDM was relatively high in this assay (difference between uninfected and infected autologous targets was less than 10%).

7.2.5 Lymphokine activated killer (LAK) cells mediate cytotoxicity

To study the non-specific cytotoxic activity seen with autologous and heterologous uninfected MDM mediated by LAK cells, PBL were cultured with rhIL2 (10U/ml) for 5-13 days and then used as effector cells (LAK). MVV-infected and uninfected MDM were set up as target cells. These results showed various levels of non-specific lysis of MVV-infected or mock-infected targets (autologous and heterologous) in different sheep (figure 7.5 A, B, C). Autologous MVV infected MDM were more susceptible than other targets with sheep 647R (figure 7.5A). Sheep 647R was MVV infected and is known to have precursor CTL in its blood which may be causing this result, however sheep 1138W (figure 7.5 B, C) is uninfected and precursor CTL have never been detected in the blood of uninfected sheep. All MDM targets were lysed to the same extent with LAK cells from sheep 1138W. Therefore the reactivity shown by IL-2 stimulated PBL for MDM is likely to be LAK mediated rather than CTL mediated. The susceptibility of macrophages

Figure 7.3 Stimulation of CTL by macrophages *in vitro*

PBL were stimulated on MVV-infected autologous MDM (A: sheep YT40) or AM (B:1257J) in the presence of IL-2 for 2 weeks as described in section 2.10.1. Target cells (1×10^4 per well), autologous and heterologous MDM (A & B) as indicated, in 96-well flat-bottom microtiter plates were infected or mock-infected with MVV, 1 TCID₅₀ per cell (MDM), for 2 days. Cells were labeled with 1.5 μ Ci ⁵¹Chromium in the well overnight before being washed and used in cytotoxic assays (16 hours) which were performed at 2 or 3 different target : effector ratios as indicated.

Results have been expressed as % specific ⁵¹Cr release. The percentage spontaneous ⁵¹Cr release from targets compared to maximal release was.: A: 25 - 31%; B: 31-32%.

FIGURE 7.3

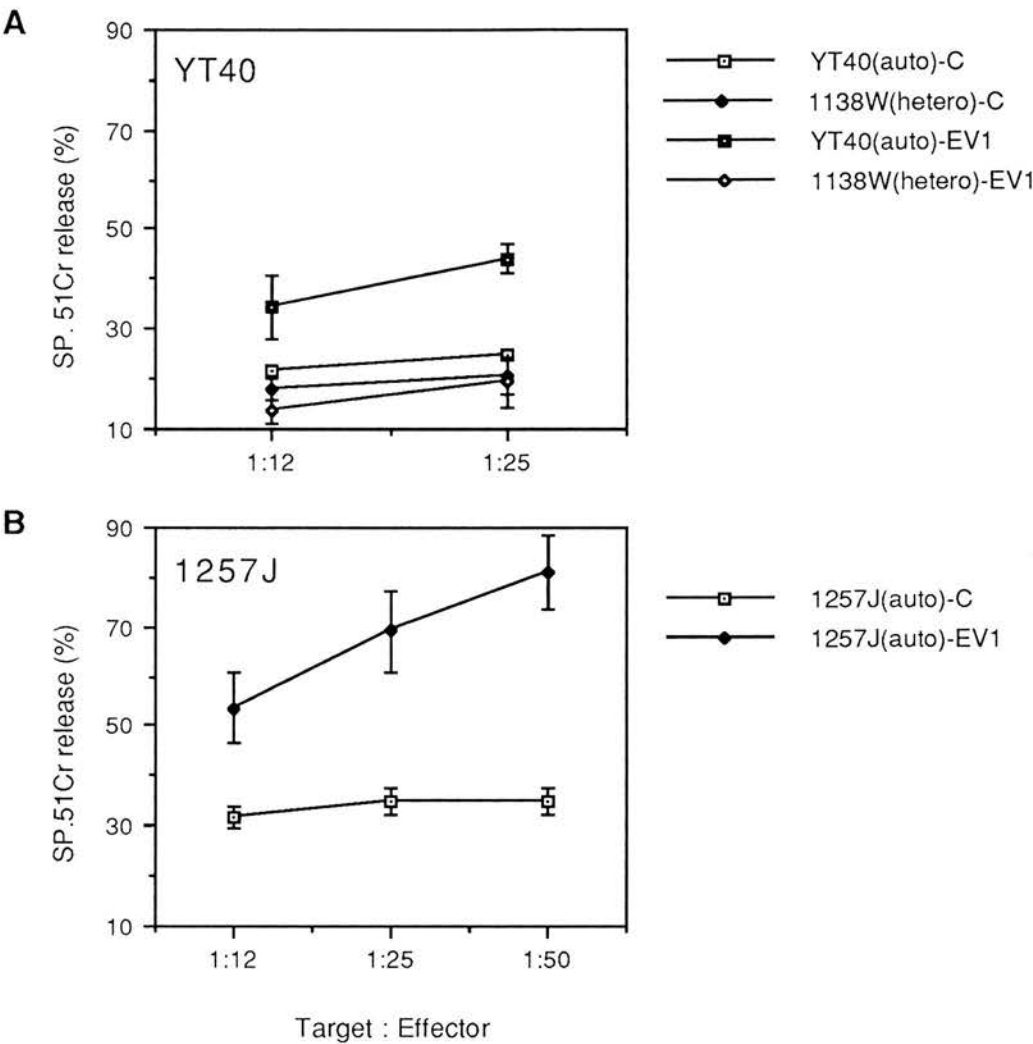
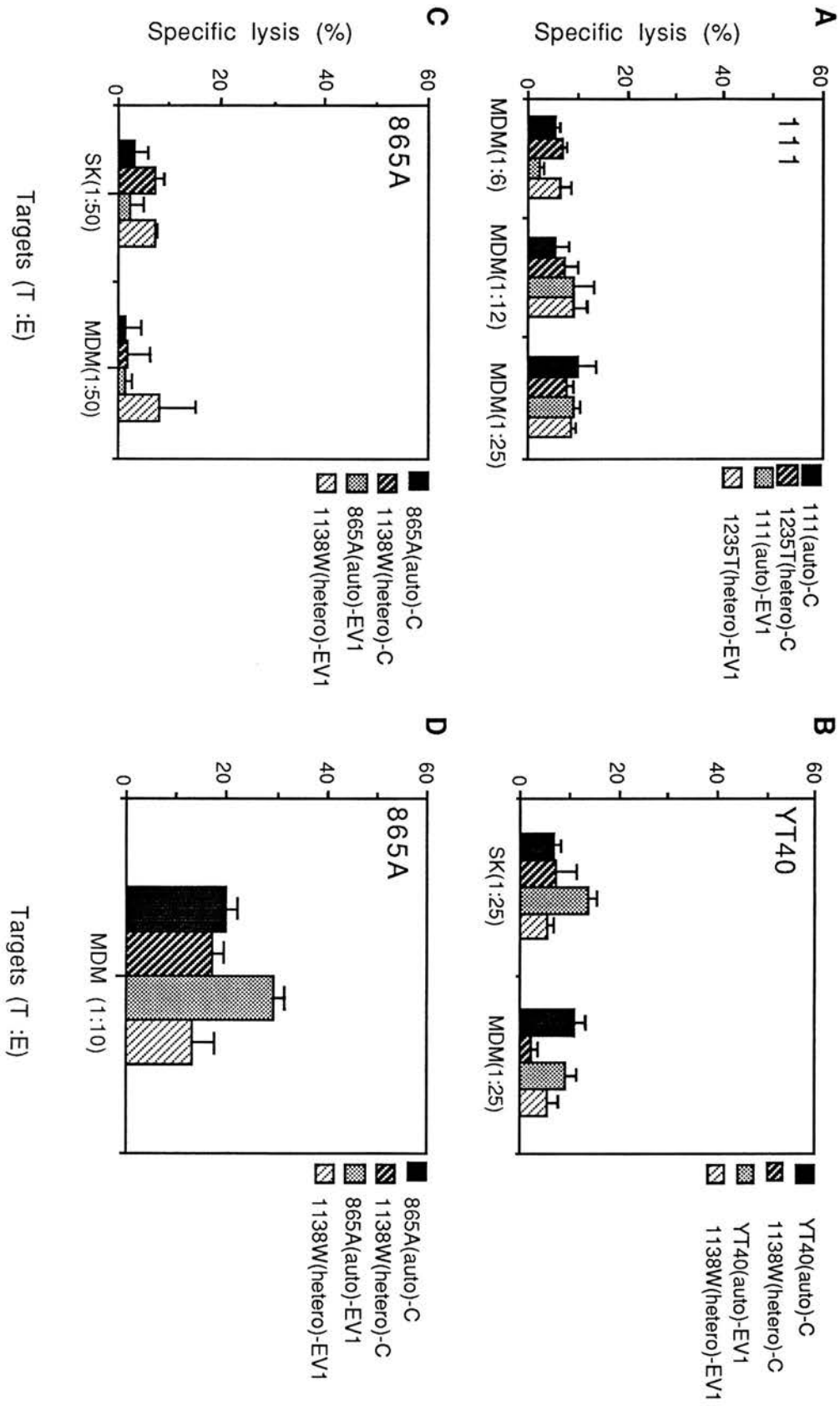


Figure 7.4 Alveolar lymphocyte effectors from MVV-infected sheep

Alveolar lymphocytes were purified from BAL fluid from 3 MVV-infected sheep (A: 111; B: YT40; C: 865A) by adherence of AM to plastic plates or tissue culture flasks twice. The AM-depleted fresh alveolar lymphocytes were used as effector cells (A, B, C). Alveolar lymphocytes from sheep 865A, were also stimulated on autologous MVV-infected skin fibroblasts (SK) for 12 days as described in section 2.10.1 and then used as effector cells (D). Target cells (1×10^4 per well), autologous and heterologous SK or MDM (as indicated), in 96-well flat-bottom microtiter plates were infected or mock-infected with MVV, 0.5 TCID₅₀ (SK) or 1 TCID₅₀ (MDM) per cell, for 1 (SK) and 2 days (MDM), respectively. Cells were labeled with 1.5 μ Ci ⁵¹Chromium in the well overnight washed and then used in the cytotoxic assay (16 hours) using different target and effector ratios as indicated.

Results have been expressed as % specific ⁵¹Cr release. The percentage spontaneous ⁵¹Cr release from targets compared to maximal release was: A 22-27%; B: 20-23%; C: 28-31%; D: 26-29%.

FIGURE 7.4



to lysis was maturation dependent; immature MDM (day 5) were more resistant than mature MDM day (12-14) (data not shown). Skin fibroblasts, especially MVV-infected cells, were also lysed by LAK cells, but the levels were relatively low compared to MDM targets (figure 7.5.C).

NK (LAK) cells in humans express CD2 and CD16 and it is thought these molecules that cytotoxic activity is triggered through these molecules (Segal and Snider, 1989). CD16 is the Fc γ III receptor for which there is no specific monoclonal antibody in sheep. FcR⁺ cells were separated from others in PBL by their ability to bind antibody coated RBC. Erythrocyte rosetting (ER) positive (ER⁺) and negative (ER⁻) PBL were separated (section 2.10.5), cultured in RPMI medium with IL-2 for 5 days and then used as effector cells. The results clearly indicated that ER⁺ LAK cells mediated cytotoxicity, but infected cells were not recognized to a much greater extent than uninfected cells (figure 7.6).

7.3 Discussion

Development of viral specific humoral and CMI responses, especially CTL, is important in controlling viral replication and dissemination. Cell mediated cytotoxicity is predominantly mediated by CTL and NK cells under MHC-restricted and non-MHC-restricted conditions respectively (Berke, 1983; Segal and Snider, 1989). They normally recognize altered antigen on target cell surfaces, for CTL this is viral peptide in MHC antigen grooves (Brodsky and Guagliardi, 1991) whilst the altered antigen for NK cells is unknown. The binding of target cells to cytotoxic cells induces the effector cell to deliver a "lethal hit" to the bound target cell causing it to die (Segal and Snider, 1989). One model of killing target cells by cytotoxic cells involves the secretion of granules containing granzymes and perforin. Released

Figure 7.5 Cytotoxic activity mediated by lymphokine-activated killer cells

LAK cells were stimulated by culturing 2×10^6 PBL (monocyte depleted) per ml (A MVV infected sheep 647R; B & C uninfected sheep 1138W) in RPMI/10%FCS in the presence of rhIL-2 (10U/ml) for 5 days (A, B) and 13 days (C), respectively. Target cells (1×10^4 per well), autologous and heterologous MDM (A, B, C) and autologous skin fibroblasts (SK) (C) in 96-well flat-bottom microtiter plates were infected or mock-infected with MVV, 0.5 TCID₅₀ (SK) or 1 TCID₅₀ (MDM), for 1 (SK) and 2 days (MDM) per cell, respectively. Cells were labelled with 1.5 μ Ci ⁵¹Chromium in the well overnight, washed and then used in cytotoxic assays (16 hours) with different target : effector ratios as indicated.

Results have been expressed as % specific ⁵¹Cr release. The percentage spontaneous ⁵¹Cr release from targets compared to maximal release was: A: 27-31%; B: 26-30%; C: 18-25%.

FIGURE 7.5

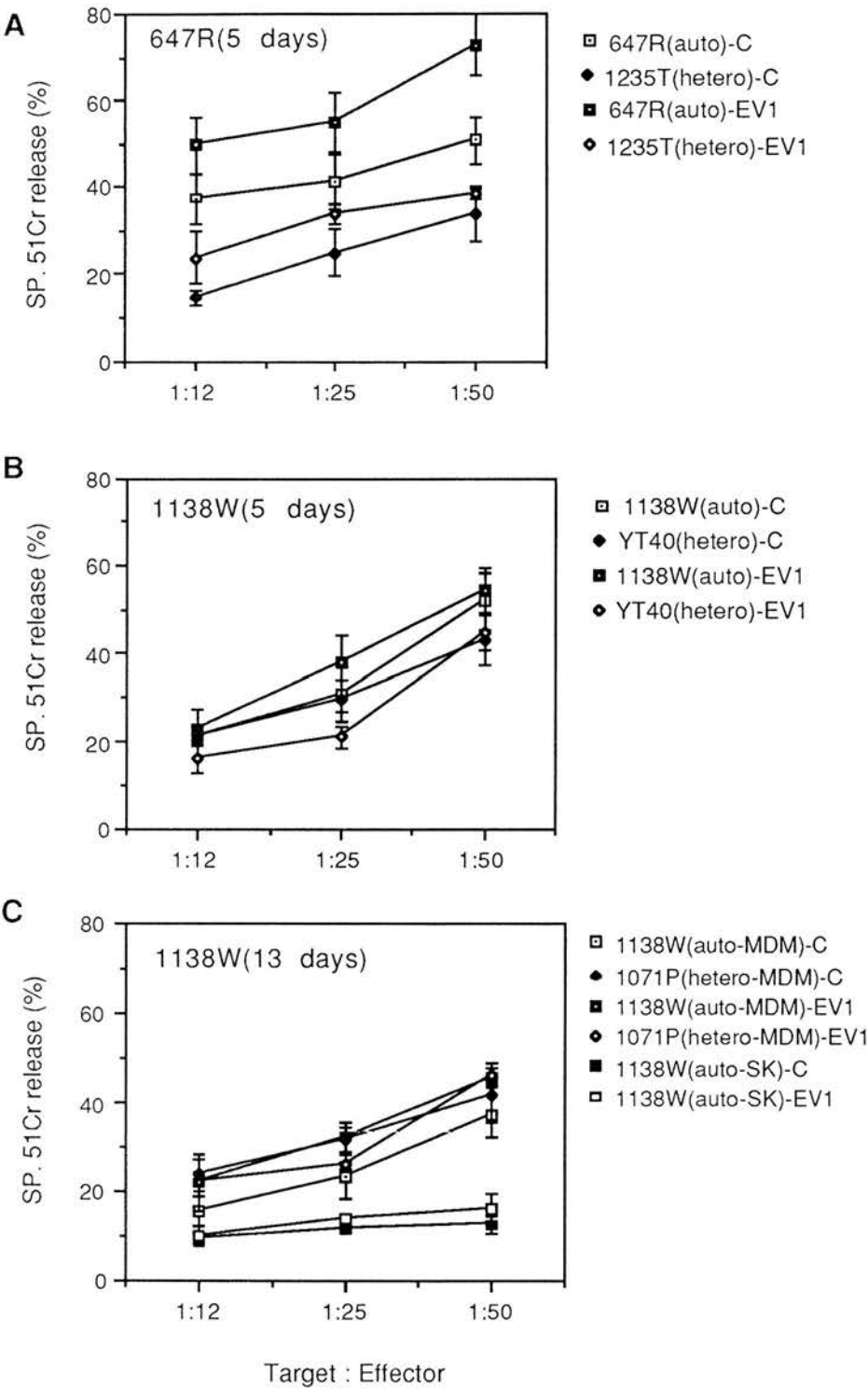
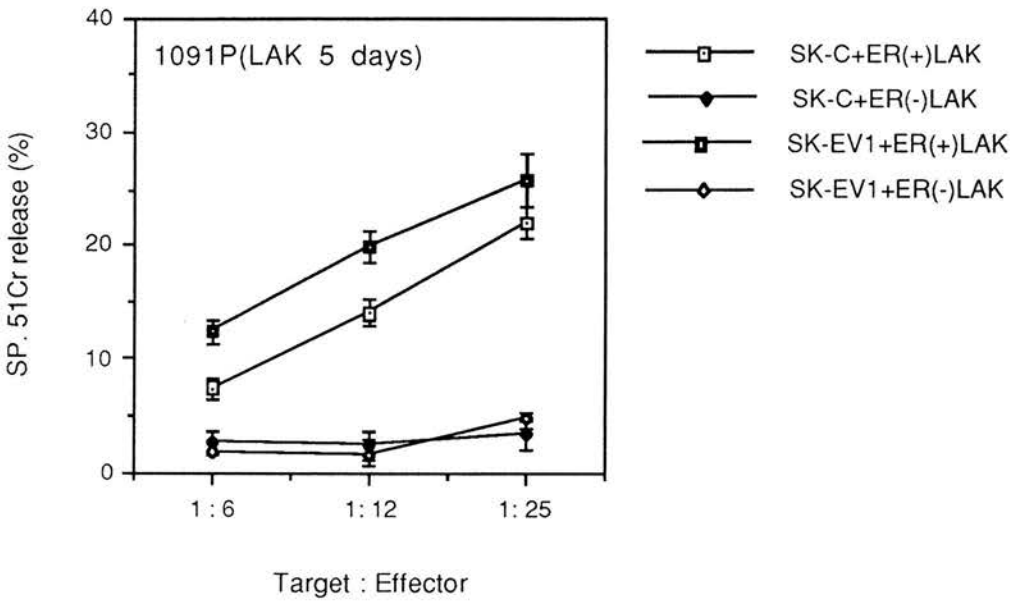


Figure 7.6 Erythrocyte rosetting positive LAK cell mediated cytotoxicity

Erythrocyte rosetting positive (ER⁺) and negative (ER⁻) PBL (MVV-infected sheep 1091P) were separated as described in section 2.10.5 and then cultured in the presence of rh-IL2 (10U/ml) for 5 days before use as effector cells. Target cells (1x10⁴ per well) were autologous skin fibroblasts (SK) infected or mock-infected with MVV, 0.5 TCID₅₀ per cell for 1 day. Cells were labeled with 1.5 µCi ⁵¹Chromium overnight, washed and then used in cytotoxic assays (16 hours) with different target : effector ratios as indicated.

Results have been expressed as percentage specific ⁵¹Cr release. The percentage spontaneous release was 22-25% of the maximal release.

FIGURE 7.6



perforins polymerize to form polyperforins, insert into the membrane to create transmembrane channels and then trigger target cell death, apoptosis (Cohen, 1991).

CTL activity in lymphocytes not given *in vitro* stimulation is rare in PBMC from MVV-infected sheep (Blacklaws et al., 1994), even in lymphocytes draining acutely infected lymph nodes (Bird et al., 1993). Additional stimulation of precursor cells *in vitro* is necessary to see CTL activity in most MVV infected sheep. This was also found with CAEV, the most closely related lentivirus to MVV; goat PBL had to be stimulated with viral antigen *in vitro* to see CTL activity (Lichtensteiger et al., 1993). However in 1/7 sheep CTL were demonstrated in uninfected PBMC. In HIV, 15-85% patients may show killing without secondary *in vitro* stimulation (Nixon et al., 1992) and CTL activity without *in vitro* stimulation has also been reported in PBMC with SIV (Vowels et al., 1989). Both HIV and SIV replicate in PBMC and it was therefore thought that if lymphocytes from a major site of MVV replication were studied, sufficient antigen may be presented *in vivo* at these sites to stimulate CTL activity which could then be seen directly *in vitro* in a higher proportion of cases. None of the sheep analyzed showed clear CTL activity in alveolar lymphocytes (Figure 7.4). However, none of the lungs obtained showed gross lesions and therefore may not have been the best samples to use: clinical scoring of sheep for maedi is difficult and inaccurate and advanced maedi cases usually do not develop due to management and control of experimental sheep. There is no significant increase in the number of CD8⁺ lymphocytes in the BAL fluid of sheep without lung lesions and the number of lymphocytes recovered from these lungs was also low and so effector to target ratios were low.

In contrast, direct CTL have been reported in alveolar lymphocytes without stimulation in HIV-infected patients with interstitial pneumonia at low E: T ratios (Plata et al., 1987). HIV-infected patients with interstitial pneumonia display an abnormally high infiltration of CD8⁺ D44⁺ T lymphocytes (Plata et al., 1987) which suggests these cells are a subset of CTL. These patients also showed high

levels of AM (12-58%) expressing viral antigen (Plata et al., 1987). It is these viral antigen expressing AM which may be stimulating alveolar lymphocytes to become active CTL. However, a high frequency of AM expressing viral antigen is not seen in MVV infected sheep without lung lesions (Watt et al., 1992b; personal observation). In a similar manner to the results shown here, direct CTL activity mediated by alveolar lymphocytes cannot be detected in HIV seropositive patients without interstitial lung lesions (Autran et al., 1991). It has been reported that the number of CD8⁺ lymphocytes in MVV-infected sheep with pulmonary lesions is increased and that most alveolar lymphocytes are also activated (Cordier et al., 1992; Watt et al., 1992b). Hence, the results shown here cannot be extrapolated to show lack of CTL activity in lungs in MVV-infected sheep with pulmonary lesions.

A subpopulation of HIV-infected patients with AIDS develop suppressor T lymphocytes (CD8⁺, CD57⁺) in BAL fluid which suppress CTL activity (Joly et al., 1989; Autran et al., 1991). The decrease of cytotoxic activity in HIV-infected patients with AIDS is also related to the gradual depletion of CD4⁺ lymphocytes and the impairment of cytokine production by CD4⁺ lymphocytes (Murray et al., 1984). Whether there is a development of suppressor T lymphocytes which suppress CTL activity in MVV infection is still not clear.

Precursor CTLs in the blood of MVV infected sheep were stimulated consistently by autologous MVV infected AM and MDM. Previous studies have used skin fibroblasts (Blacklaws et al., 1994; Lichtensteiger et al., 1993) and also MDM (Kennedy-Stoskopf, 1989) to present antigen. Therefore the *in vivo* infected cell type can stimulate precursor CTLs into active CTLs. Similarly, both AM and MDM can act as targets for MHC restricted lysis by MVV specific CTL. AM have also been used successfully as targets with HIV specific CTL (Plata et al., 1987; Autran et al., 1991). Using skin cells, the CTL population has been shown to be CD8⁺. Skin fibroblasts do not express MHC class II molecules by FACS analysis and therefore use of the MHC class I/CD8⁺ interaction is not surprising. However,

AM and MDM express MHC class II especially when cultured with lymphocytes and so may be useful targets in determining the role of class II restricted CD4⁺ CTL in MVV. CD4⁺ CTL have been reported in HIV infected and in antigen primed situations (Orentas et al., 1990; Littaua et al., 1992; Curiel et al., 1993).

Skin fibroblasts gave lower backgrounds and variation and better viral infections than AM and MDM and so results were much clearer with fibroblasts. Here there was often recognition of uninfected autologous macrophages which confused results. Depletion of CD8⁺ cells caused a decrease in recognition of MVV infected autologous MDM but did not reduce the ⁵¹Cr release to background levels. The same CD8⁺ depletion using MVV infected skin fibroblast targets reduced cytotoxicity to background levels. Therefore the major effector population with MDM targets was CD8⁺ but other effectors may also have been present (CD4⁺). However depletion of CD4⁺ lymphocytes did not reduce the levels of lysis seen, perhaps because sufficient CD8⁺ lymphocytes remained to give maximal lysis over 16 hours.

Inflammatory lesions may induce the activation of macrophages which are then, if infected, not only targets for MVV specific CTL but also for NK or LAK cells. IL-2 has been reported to induce functionally activated LAK cells against tumor or virus-infected cells (Taylor and Cohen, 1992). IL-2 generated LAK cells also show nonspecific cytotoxic activity against macrophages, especially autologous macrophages, whether they are infected with virus or not (Djeu and Blanchard, 1988; Streck et al., 1990). Human NK cells are CD2⁺ and CD16⁺ (Perussia et al., 1984; Tilden et al., 1987; Segal and Sinder, 1989) and some NK cells also express low levels of the CD8 marker (Tilden et al., 1987; Perussia et al., 1984). Hence CD8⁺ depletion of effector cells may not only have depleted CD8⁺ CTL but also LAK cells which may be why this depletion also greatly decreased the background on non-infected MDM (table 7.2). Using erythrocyte rosetting to separate FcR⁻ and FcR⁺ cells it was found that non-specific lytic activity restricted in the FcR⁺

fraction of PBMC (figure 7.6). This would again suggest that poor background using macrophage targets were caused by FcR⁺ LAK cells.

In vivo, IL-2 administration has been found to enhance NK cell function (Djien et al., 1988; Taylor and Cohen, 1992). The lymphokine activated killers (LAK) lyse normal autologous macrophages. This phenomenon is thought to be a feedback regulatory mechanism for controlling cytokine production from activated monocyte/macrophages (Djeu and Blanchard, 1988), because over production of TNF- α or IL-1 from activated macrophages may be indirectly involved in autoimmune disease. Therefore, LAK activity may play a role in reducing the number of activated macrophages after infection.

LAK cytotoxic activity seen also varies with the kinetic activation of PBL and the maturation state of MDM as well as individual variation (Djeu and Blanchard, 1988). The frequency of LAK cells in PBL culture stimulated by MVV-infected skin fibroblasts with IL-2 may be different from PBL cultures with IL-2 for 2 weeks only. Hence it is difficult to evaluate the levels of non-specific activity involved in CTL assays here. An ADCC assay in HIV infection using U937 (monocytic cell line) as target cells showed around 35% non-specific lysis with un-infected MDM (Goudsmit et al., 1988), but the reason has not been discussed. Non-specific backgrounds have also been reported with macrophages lysed by IL-2 activated effector cells (Langlade-Demoyen et al., 1988) and with autologous non-pulsed (hsp) monocytes during a CD4⁺ mediated CTL assay (Ottenhoff et al., 1988). These reports support the view that the results obtained here with MDM targets were within normal ranges.

It has been reported that two distinct effectors mediate SIV *env*-specific target cell lysis (Yamamoto et al., 1990). One is CD16⁺, MHC class I-unrestricted which is similar to LAK cells. Weinhold et al (1988) also showed that CD4⁺ lymphocytes coated with HIV gp120 were lysed by a population of non-T cell effectors. This cytotoxicity was enhanced after activation of effector cells with

IL-2, but greatly decreased after the depletion of CD16⁺ cells. Riviere et al. (1989) using a monoclonal antibody for CD3⁺ incubated with effector cells to block reactions, inhibited *gag*-specific cytotoxic activity, but not *env*-specific cytotoxicity. Furthermore, the depletion of CD8⁺ cells had no effect on cytotoxic activity against autologous target cells infected with vaccinia virus expressing *env* protein. This suggests that two distinct types of effector cells mediate killing of targets expressing *gag* and *env* protein. NK or LAK cells play a significant role in controlling *env* protein expressing cells whilst classical CTL kill *gag* expressing cells. Here, the cell lysis seen with virus-infected autologous MDM mediated by LAK from MVV infected sheep is higher than mock-infected controls which may be due to an *env* specific effect as seen with HIV and SIV, although some CTL activity cannot be ruled out.

Cultured MDM from MVV-infected sheep, especially long-term or naturally infected sheep with pulmonary lesions, showed some differences from uninfected sheep (section 3.2.2). More cells in these cultures underwent "programmed cell death" (PCD) (Cohen, 1991; Mangan and Wahl, 1991; Ohno et al., 1993) due to expression of some early signals for death. It has been thought that PCD in monocytes may be regulated by a complex network of cytokine-mediated intercellular communication (Mangan and Wahl, 1991). This may have contributed to the higher spontaneous ⁵¹Cr release seen with of MDM compared to skin fibroblasts.

7.4 Summary

MVV-specific CTL precursors were detected in PBL of all MVV-infected sheep. MVV infected MDM and AM were targets for CTLs and could also stimulate CTL activity *in vitro*. The major effector cell population using MVV infected macrophage targets was CD8⁺ although another population may also have been involved.

There was no direct or precursor CTL activity found in alveolar lymphocytes from MVV infected sheep without lung lesions.

Effectors causing nonspecific lysis of target cells were FcR⁺ LAK cells.

CHAPTER 8

DISCUSSION

8.1 Introduction

All the lentiviruses cause persistent infections which are typified by slow disease progression. Although the depletion of CD4⁺ lymphocytes is a central feature of immunosuppression in HIV, SIV and FIV infection (Narayan and Clements, 1989; Pedersen et al., 1989; McEntee et al., 1991), macrophages are thought to be the site of viral persistence. In MVV and CAEV macrophages are also the site of viral persistence as they are the major target cells for infection. Therefore a better understanding of the interaction between MVV and macrophages may lead to more insight into the interaction of HIV and macrophages unclouded by interference by the CD4⁺ infection.

The research undertaken here looked at the interaction of MVV and macrophages with 3 main aspects: MVV replication in the macrophages; the effect of MVV infection on the phenotype and functional capacities (phagocytic and antigen presenting function) of macrophages, and the ability of MVV infected macrophages to act as targets for effector mechanisms involved in clearing virus infected cells eg CTL.

8.2 Interaction of Macrophages and Lentiviruses

Mammalian animals normally have both non-specific (NK cells, neutrophils and macrophages) and specific (CMI and humoral immunity) defences against invading pathogens. During the early phase of primary infection viruses rapidly replicate in target cells and cause lesions with a lot of progeny being produced and spread. It is at this first stage that non-specific immune mechanisms are important in limiting the amount of virus produced. Once sufficient inflammation and antigen have been produced the host develops specific immunity which is important in eliminating these pathogens and allowing recovery from infection. However for patients with defects in immunity, infection may result in death. This basic model is normally seen in most viral infections (Sissons and Oldstone, 1980). HIV, SIV and

FIV have a similar pattern in early viral infection, even though these events progress slowly compared to other viruses. There is a rapid rise in viral production in PBMC and plasma virus titer prior to the development of viral antibodies. After the development of viral specific antibodies and cytotoxic cells, most virus and virus infected cells are eliminated. However, a reservoir of viral replication is established in macrophages throughout the asymptomatic period of infection. Another peak of viral production is seen in the late phase of HIV infection when immunodeficiency becomes marked (Miedema et al., 1990; Dear et al., 1991; Clark et al., 1991). Surprisingly, there is only a low level of virus generated in acute MVV infected lymph nodes (Dr.P. Bird, personal communication) and few virus associated cells are released in efferent lymph (11 in 10^6 efferent cells). However both humoral and cell mediated immune responses are induced as is seen in other lentivirus infections (Bird et al., 1993).

In MVV infection there is a low level of viral infection (Gendelman et al., 1985), which persists together with a large amount of pathology accumulating in lung, brain, joints and mammary glands (Cutlip et al., 1979; Oliver, et al., 1983). The virus is known to persist in macrophages. Macrophages have a short life span and may die after activation and virus infection. Here it has been shown that MVV-infected macrophages can be killed by CTL and LAK cells (section 7.2) once productive infection is switched on. This is one mechanism by which productively infected cells may be cleared. Therefore the maintenance of infection in the macrophage pool requires continual recruitment of newly infected cells or latently infected cells into target tissues. There are several ways in which this may occur. Promonocytes containing proviral DNA are found in the bone marrow and may transfer infection into target organs as monocytes seed into tissues (Gendelman et al., 1985). Virions accumulating in vesicles (section 3.2.3 & 3.2.4) may be released when macrophages die or are killed by immune effector mechanisms and may be immediately taken up by neighboring macrophages. In addition, virions or viral

DNA may be passed to new daughter cells if macrophage division occurs as seen in the joints of CAEV infected goats (Jutila and Banks, 1988). This allows the equilibrium to be maintained between clearance and infection such that infection continues at low levels in target tissues.

The tissue macrophages from lesions appear activated (by surface antigen expression, fibronectin and neutrophil chemotactic production; Cordier et al., 1990) during MVV infection. The interaction of virus infected macrophages and lymphocytes at these sites may induce the secretion of cytokines, eg LV-IFN (Narayan et al., 1985; Zink et al., 1987; Lairmore et al., 1988) which amplify surface molecule changes and functions of macrophages (Cordier et al., 1990; Lujan et al., 1993). Cytokines produced from lymphocytes interacting with these activated macrophages (TNF- α and IL-1) may also enhance expression from the viral LTR and increase viral antigen production eg *env* gp110 (Clements et al., 1979). If this occurs, free viral antigen may interfere with uninfected cell functions. *Gag* p25 antigen has been seen in efferent lymph plasma (Bird et al., 1993) and so free viral antigens may be present in the body fluids of MVV infected sheep. In HIV, *env* gp120 secreted from infected cells is known to interfere with the activity of uninfected cells (Habeshaw et al., 1990; Wagner et al., 1992). The *env* protein production in MVV-infected AM *in vitro* appears normal (section 3.2.8) and although there are no reports of MVV *env* adversely affecting immune functions, this may be through a lack of research as no recombinant *env* gp110 is available. Free virus antigen may also add to the general immune activation, if macrophages take it up and present it to lymphocytes.

Whether the persistently infected macrophages at sites of MVV induced lesions (lung, synovium) are actually activated is not clear. Studies on phagocytosis and antigen presenting function in productively infected macrophages *in vitro* (section 5.2 and section 6.2) have shown functional defects. But *in vivo* there are only a low percentage of productively infected macrophages. This may be one of

the reasons why functional defects of macrophages cannot be seen *in vivo*, although a suggestion of a decrease in phagocytosis and erythrocyte rosetting may be seen in AM from lungs with lesions (section 5.2.6).

8.3 Immunosurveillance problems

Another problem with lentivirus infections is that virus is not cleared by host immunosurveillance. Factors contributing to control of viral replication *in vivo* may include: virus-specific CTL and antibodies, non-specific NK (LAK) cells, IFN- γ , and macrophages themselves. Factors allowing persistence of the virus include the restriction of viral replication, antigenic drift (Narayan and Clement, 1989) or perhaps the induction of the wrong types of effector mechanisms.

Restriction of viral replication and persistence in target cells are characteristics of lentiviral infection. Only a few cells are infected *in vivo* and these contain relatively low copy numbers of viral RNA (50-150 copies per cell). In contrast, *in vitro*, there are thousands of copies of the viral genome and more than 50-100 progeny virions are produced per cell (Haase et al., 1982). Viral persistence has been found in lung, brain, spleen and lymph nodes, but there is no evidence showing MVV persistence in Kupffer's cells (Gendelman et al., 1985; Kennedy et al., 1985), the resident liver macrophage. Hence, it is possible that within the liver microenvironment, a host regulatory factor within Kupffer's cells is regulating monocyte differentiation and is aborting viral infection in Kupffer's cells. The study of the interaction between MVV and Kupffer's cells, whether virus can enter the cell and if so, how this infection is aborted by the Kupffer's cell, may be helpful in finding a way to breakdown viral persistence in macrophages. The block in infection of Kupffer's cells has been overcome by EIAV (Salinovich et al., 1986), another lentivirus where Kupffer's cells are the major target cells. However, *in vitro* observations may not reflect what happens in a much more complex *in vivo* situation in which the immune system is involved.

Recently, using PCR with *in situ* hybridization, a high frequency of target cells harboring viral DNA have been shown in the lung (Staskus et al., 1991; Haase et al., 1990). These epithelial cells did not have viral RNA and so may have been truly latent. Latent cells do not express viral antigen and so cannot be removed by immune effector mechanisms allowing virus to persist in the host.

Antigenic mutation may allow MVV to persist in sheep. Escape mutants are known to arise in MVV to neutralizing antibody and in HIV, CTL escape mutants have also been documented (Narayan et al., 1978; Phillips et al., 1991). Again this would allow the persistence of virus in the host. Early work on immune responses to MVV show transient proliferative responses to MVV antigen (Larsen et al., 1982; Shivonen et al., 1981) which would also allow periodic replication of the virus in the host. However recently, it has been reported that proliferation to MVV antigen (Reyburn et al., 1992b) and CTL activity (Blacklaws et al., 1994) can be consistently detected in PBMC from MVV-infected sheep. Additionally, antibody is always present; as no viremia is seen in MVV, neutralizing antibody may be functioning all the time. We have shown that macrophages can act as targets for CTL and can induce CTL activity (section 7.2). All these suggest that good immunosurveillance is in operation. This may be why little antigen and few infected cells are seen at lesions. Virus would therefore need to persist by other mechanisms eg latency.

It is not known which MVV antigens are recognized by CMI responses (except p25 in the proliferation response; Reyburn et al., 1992b). In HIV and SIV nearly all viral antigens are recognized (Michel et al., 1992; Letvin et al., 1992). Lentivirus replication has an early stage in which non-structural proteins involved in control of virus replication are expressed (*rev* and *tat*). Later productive stages of virus replication probably have expression of all viral genes including the structural genes (*gag*, *pol*, and *env*). Good immunity should involve responses to early antigens ie *rev* and *tat* which would allow viral replication to be stopped before

progeny are produced. It will be interesting to see if in MVV, there are good immune responses (CTL) to *rev* or *tat*, as this may be one of the most important factors in keeping the numbers of infected cells low *in vivo*. MVV has a restricted replication state in monocytes (Gendelman et al., 1986), but it is not known if *rev* and *tat* are expressed in these infected cells, and so whether these can be targets for immune surveillance. If it was shown that there was a specific lack of immunity to early viral antigen it would be very interesting to see if immunization could overcome this defect and if this helped to decrease the numbers of infected cells seen *in vivo* ever further.

CD8 proliferation is a common feature in many viral infections (McMichael et al., 1983) and lentiviruses are not exceptions. A great increase in CD8⁺ lymphoblasts was seen in efferent lymph after MVV infection, but the CTL activity detected in 1 out of 5 sheep during this period was when cell-associated virus exited the lymph node (Bird et al., 1993). A similar phenomenon, activated CD8⁺ lymphocytes, was seen in lymph nodes of monkeys after SIV infection (Reimann et al., 1991). These CD8⁺ lymphocytes are activated, but do not express IL-2 receptor (CD25) and so are thought to be in a defective maturation state (Bird et al., 1993; Reimann et al., 1991; Salazar-Gonzalez et al., 1985). An increase in CD8⁺ lymphocytes is also seen in bronchoalveolar lavage, CSF or synovial fluid from MVV infected sheep, particularly in those sheep which have developed lesions (Harkiss et al., 1991; Torsteinsdottir et al., 1992; Lujan et al., 1993; section 4.2.7). Though virus-specific CD8⁺ CTL are important in the lysis of virus-infected cells during acute infection, the activated CD8⁺ lymphocytes in lentiviral infection may mainly function to regulate viral replication. The increase in CD8⁺ lymphocytes does not parallel CTL activity in SIV and MVV (Reimann et al., 1991; Bird et al., 1993), but activated CD8⁺ lymphocytes can suppress HIV and SIV replication in CD4⁺ lymphocytes in a non-MHC restricted manner (Kannagi et al., 1988; Walker et al., 1988). This point has not been studied in MVV infection.

Evidence shows that there is only an IgG₁ response to MVV antigen after viral infection (Reyburn, 1992a), but IgG₂ responses can be induced by immunizing with *gag* p25 antigen (Dr. P. Bird, personal communication). The presence of Th1 or Th2 lymphocytes and which cytokines mediate the production of IgG₁ or IgG₂ has not been shown in sheep, but there may be a predominant antibody isotope produced due to the induction of a specific helper cell subset (Th2). IgG₁ antibody in MVV infected sheep may not mediate ADCC activity (Reyburn, 1992a). This may be an important defect in control of viral replication. If the other subpopulation (Th1 lymphocytes) was induced would a different and more effective antibody be produced? The induction of incorrect immune effector mechanisms eg IgG₁ and CD8⁺ cells discussed above may fail to efficiently eliminate virus infected cells and allow lentivirus persistence.

Most previous studies on MVV infection used PBMC and serum because of ease of sampling. There is no significant alteration in lymphocyte phenotypes in PBMC and good antibody and CMI responses appear to be present in infected sheep. However, there are significant changes in CD4:CD8 lymphocyte ratios and lymphocyte and macrophage phenotypes in lung, synovium and CSF (Cordier et al., 1992; Lujan et al., 1993; Torsteinsdottir et al., 1992), suggesting that the situation in blood may not really reflect the situation in organs targeted by MVV infection. For example we cannot prove CTL activity in BAL fluid (section 7.2.4) whilst it was clearly seen in PBMC. This may be due to the wrong specimens (samples from lungs with no lesions) or low effector : target ratios being used, but this could also indicate a specific lack of immunity in sites where virus is found.

8.4 Respiratory Tract Immunity

Concurrent infection with pasteurellosis is a common feature in maedi, especially in field cases (Cutlip et al., 1977; Markson et al., 1983; Myer et al., 1988), but it is not seen in MVV infected sheep without lung lesions, suggesting that the immunological defence of the respiratory tract may be impaired in some sheep which have developed pulmonary lesions. Pulmonary defence against infection is very complex and consists of local humoral (IgA and IgG) and cellular immunity, complement, ciliary activity, mucus production and phagocytosis (Burrells, 1985; Christensen and Mousing, 1992). The major immunoglobulin generated in the respiratory tract particularly in the upper respiratory tract is secretory IgA which can efficiently agglutinate invading bacteria; this may enhance mucociliary clearance of pathogens and inhibit bacterial colonization of the mucosa. However significant amounts of IgG and complement components are also found in the lower respiratory tract (Burrells, 1985). These plasma components enter the respiratory system by transduction, although some may be produced locally (Kaltreider, 1976). IgG and complement components are an important source of opsonin for phagocytes. The phagocytic and microbicidal effect of AM and the opsonins in the pulmonary environment therefore act together in destroying invading pathogens (Kaltreider, 1976). In maedi affected lungs, the alveolar walls are greatly thickened which may decrease the transduction of plasma factors into the lung. This would reduce the amount of opsonin in the respiratory system available for action with AM, allowing the replication of gram negative bacteria (which require opsonization for phagocytosis) entering alveolar areas. The impairment of CD4⁺ lymphocyte function and production of cytokines (IFN- γ) which decrease AM function contribute to opportunistic pathogen infections seen in AIDS patients. There is no evidence of mycotic and protozoal infections (phagocytosed without opsonization) in MVV infected sheep (Markson et al., 1983), but these are often seen in AIDS patients (Marchevsky et al., 1985). This may indicate that the basic phagocytosis of

fungi and protozoa by AM from MVV-infected sheep is intact. Indeed the absence of a significant difference in the phagocytic activity of AM from MVV infected sheep with pulmonary lesions compared to controls supports this view (section 5.2.6). In addition, increases in the number of activated lymphocytes in MVV have been reported (Watt et al., 1992b; Cordier et al., 1992). The cytokines secreted from activated lymphocytes may activate macrophage functions (phagocytic and intracellular killing activity) which are important in eliminating pathogens. Therefore the physiological impairment of the lung (thickness of alveolar walls) may be more important in allowing gram negative bacterial superinfection in maedi affected lungs than alterations in AM function.

Recently, research has focused on the suppressive effect of AM, via nitric oxide, on pulmonary immunity (Mills, 1991; Holt, 1986). Nitric oxide is functional against microorganisms and tumor cells (Liew and Cox, 1991), but also suppresses pulmonary DC function and lymphocyte responses (Holt et al., 1986 & 1993). One hypothesis suggests that the suppressive effect of AM is necessary for the control of the non-specific activity of AM. AM frequently come into contact with foreign antigens from air or the upper respiratory tract which may non-specifically trigger AM to release accessory cell factors leading to immunopathological lesions. The major physiological function of AM is thought to be the release of chemotactic factors, ingestion and degradation of antigen and microbicidal activity (Brain, 1992) rather than an antigen presenting function for lymphocytes. Therefore, the increase in the number of lymphocytes and AM and their activation in MVV infection is not only responsible for the induction of the active chronic inflammation seen, but also may cause a suppressive state in local immunity. This may contribute to the concurrent infections seen in some maedi affected lungs.

8.5 Immune Mechanisms of Pathology

Pathology accumulated during MVV infection is mediated by immune responses (Nathanson et al, 1976). It is not known however, if this is through MVV antigens or responses to autoimmune antigens. It has been shown that CSF lymphocytes proliferate to MVV antigen (Torsteinsdottir et al., 1992). The levels of proliferation correlated to the severity of lesions, suggesting that anti-MVV immunity mediates pathology. This could be through persistent, small amounts of viral antigen being produced in target organs which continuously stimulate immune responses and cytokine production (low levels) leading to chronic inflammation.

It has been shown that stressed macrophages, either virally infected or cytokine activated, may induce heat shock protein (hsp) expression which can be recognized by $\gamma\delta$ T cells (Aquino et al., 1992) or NK and LAK cells (section 7.2.5). Hsp is expressed on HIV-infected lymphocytes which are lysed by hsp-specific antibody (ADCC) (Cesare et al., 1992), cytotoxic CD4⁺ lymphocytes (Munk et al., 1989; Ottenhoff et al., 1988) and NK cells (Cesare et al., 1992; Djeu and Blanchard, 1988). Hence, it is possible that MVV-infected macrophages may also express hsp antigen (Dr G. Harkiss, personal communication) and initiate autoimmune responses leading to chronic inflammation.

8.6 Summary

My work has looked at the *in vitro* infection of macrophages with MVV. In this system, most macrophages become productively infected. This is different to the situation *in vivo* where low frequencies of productive virus infection are seen. MVV infection does appear to decrease antigen presenting and phagocytic functions of macrophages *in vitro* which you would expect to lead to immunosuppression. However, immunosuppression is not usually seen *in vivo*, due to the low levels of infection and the presence of other cell types which can take over these functions. Infected macrophages are targets for immunosurveillance (CTL and NK/LAK cells)

and the low levels of infected cells and antigen seen *in vivo* may be, in part, due to clearance from the host and so mechanisms for evasion of the immune response must exist. These may include latency or restricted replication in immature macrophages.

In vivo a complex interaction with lymphocytes and macrophages causes a chronic inflammatory state which was not replicated here by the *in vitro* infected macrophages. Future work should therefore perhaps focus on the production of sheep promonocyte/monocyte cell lines or the definition of *in vitro* culture conditions which would allow the establishment of persistent MVV infections *in vitro* and their effect on macrophage function to be studied. From results shown here, functional assays with *in vivo* material (AM, synovial macrophages) should be continued as the changes in phenotype documented do not necessarily correlate to changes in macrophage function.

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